CLONING AND CHARACTERIZATION OF THE MOUSE HEPATITIS VIRUS RECEPTOR

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ABSTRACT

Title of dissertation: Cloning and Characterization of the Mouse Hepatitis Virus Receptor

Gabriela S. Dveksler, Doctor of Philosophy, 1991

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The attachment of mouse hepatitis virus (MHV), a coronavirus, to the host cell membrane is the key first step leading to viral infection. The cellular receptor for MHV has been previously characterized as a 100 -120 kDa membrane glycoprotein, found in colon , small intestine and liver. This receptor has been shown to be the only portal of entry for MHV-A59.

Identification of the mouse gene for the MHV receptor is essential in understanding the mechanism of host cell-virus interaction. To this end, a new cloning strategy based on the polymerase chain reaction technology was developed using RNA as starting material (RNAPCR). I employed glyceraldehyde-3-phosphate dehydrogenase as a control gene for the establishment of this cloning strategy.

Amino acid homology and antibody reactivity had pointed to the murine carcinoembryonic antigen (CEA) family as a candidate for the cellular receptor for MHV. Using the RNAPCR system with information obtained from the partial N-terminal amino acid sequence for the MHV receptor and a partial murine

CEA cDNA sequence, a 710 bp product was obtained. Nucleic acid sequencing confirmed that this clone was a portion of the receptor. This fragment was then used as a probe to screen a BALB/c liver lambda gt11 cDNA library, from which a clone was obtained that begins at amino acid 10 and ends with a poly A tail. Using an alternative PCR technique, the sequence of the first 10 amino acids of the mature receptor protein and part of the leader peptide were then identified.

The partial MHV receptor cDNA was transcribed and translated in vitro. The in vitro synthesized protein had the predicted size based on the amino acid sequence, and was immunoprecipitated with polyclonal antibody directed against affinity-purified MHV receptor. This polyclonal antibody has been shown to block MHV infection of murine tissue culture cells to a dilution greater than 1/1,200.

The identified MHV receptor is a member of the CEA gene family of unknown cellular function. CEA is a subfamily in the immunoglobulin superfamily. Thus MHV, like several other animal viruses, uses a member of the immunoglobulin superfamily as a portal of entry into the cell.

CLONING AND CHARACTERIZATION OF THE MOUSE HEPATITIS VIRUS RECEPTOR

by

GABRIELA SUSANA DVEKSLER

Dissertation submitted to the Faculty of the Department of Pathology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirement for the degree of Doctor of Philosofy, 1991

To my father, Jaime I. Dveksler

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INTRODUCTION

Definition and relevance of viral receptors : A major feature of a number of viral infections is the selective damage to specific tissues, and in some instances to specific cells within a tissue. A classical example of this is the infection by poliovirus of the anterior horn cells of the spinal cord (Jubelt et al., 1980). Other examples include selective infection of the limbic system by rabies virus (Johnson, 1965), infection of ependymal cells by mumps virus (Johnson, 1968), infection of pancreatic beta cells by encephalomyocarditis virus or Coxsackie B4 virus (Craighead, 1968; Yoon et al., 1980) and infection of T lymphocytes, monocytes, epidermal Langerhans cells and cells in the brain by human immunodeficiency virus (HIV) (Klatzmann et al., 1984a; Klatzmann et al., 1984b; Gartner et al., 1986; Tschachler et al., 1987; Maddon et al., 1986).

Virus receptors are the cell surface components to which a virus attaches prior to penetration and infection. The viral moiety that attaches to cells is the viral attachment protein. The definition of a cellular receptor ultimately depends upon the structural and functional identification of a site that is specifically recognized by a ligand. In classical ligand-receptor interactions, the cell surface receptor is a protein with a binding domain that interacts with a soluble ligand. For virus-receptor interactions, the cell surface receptor is either a protein, a carbohydrate, or a lipid molecule. The ligand is the viral attachment protein. Three major criteria, derived from models of ligand-receptor interactions (Bennet, 1978), are taken into account when considering a viral recognition site

as a receptor: saturability, specificity, and competition. The first (saturability) is reflected by the fact that the virus should have only a finite number of binding sites along the cell membrane and that high concentration of virus should be able to saturate them. The second (specificity) refers to the binding of the virus to cells that the virus infects or to cells where binding induces a biologically measurable response. The third criterion (competition) is demonstrated in viral systems by the ability to block the binding of radiolabeled virus with unlabeled virus or viral attachment protein.

It has long been postulated that the in vivo tropism of viruses is mediated in part by the presence or absence of specific receptor sites on the surface of target cells (McLaren et al., 1959; McLaren et al.,1960; Holland, 1961). This concept is based on the correlation found between the ability of cells to adsorb virus and to support viral replication. Non-primate cell lines resistant to infection by intact poliovirus become infected and produced progeny virus when exposed to infectious poliovirus ribonucleic acid that is presumably taken up nonspecifically by cells (Holland and McLaren, 1959). Wilson et al., (1977) showed that virus-resistant cells become infected when the poliovirus genome is introduced via lipid vesicles. Experiments demonstrating a correlation between the presence of receptors on host cells and susceptibility to infection have been reported for many picornaviruses (Crowell and Landau, 1983; Crowell et al., 1981).

An example of tissue tropism based on the presence of cell surface receptors is found in mammalian reoviruses. While reovirus type 1 infects

ependymal cells of the brain causing ependymitis in humans and hydrocephalus in mice, type 3 infects neurons causing fatal encephalitis in mice (Margolis and Kilham, 1969; Weiner et al., 1977; Spriggs et al., 1983). Consistent with their known tissue tropisms, it has been shown using immunofluorescence techniques that reovirus type 1 binds to the ependymal cells, producing intense staining, while negligible binding of reovirus type 3 is observed (Tardieu and Weiner, 1982). Tissue tropism in this case has been mapped to the viral hemagglutinin.

Identification of viral receptors has proved extremely difficult because virus particles can adhere to many substances, including inert materials. Viruses may also adhere to cell surfaces non-specifically through electrostatic interactions (Tardieu et al., 1982). Virus particles might be internalized through non-specific mechanism such as fluid-phase or constitutive endocytosis (Steinman et al.,1983). For these reasons, it is often difficult to distinguish between non-specific binding and biologically relevant binding. Other complexities arise from the possibility that some viruses can utilize more than one type of receptor and that specific virus receptors may be present in low numbers on the cell surface or may be labile.

Although many viruses interact directly with a host cell receptor, some viruses bind indirectly to a cell via an intermediate molecule. The best known example of this type of interaction is the enhancement of virus infection by antibodies, which has been demonstrated for several viruses including dengue virus (Halstead,1988; Halstead and O'Rourke,1977), West Nile virus (Peiris et

al., 1981), and most recently human immunodeficiency virus (HIV) (Homsy et al., 1989). The Fc portion of anti-viral antibodies coating the virus interacts with cell surface Fc receptors cross-linking virions to the cell surface and facilitating uptake of the virus-antibody complex. In addition, complement-dependent enhancement of West Nile virus infectivity has been described in which the C3b component of complement binds complexes of virus and IgM to type 3 complement receptors of macrophages (Cardosa et al., 1986) . Similarly, polymerized serum albumin can act as an intermediate receptor for hepatitis B virus by binding to polymeric albumin receptors on hepatocytes (Machida et al., 1984). Mediation of virion penetration by association with cellular proteins has been suggested for herpes simplex virus type1 (HSV-1). HSV-1 recognizes the basic fibroblast growth factor (FGF) receptor by having basic FGF associated with the virion (Baird et al., 1990).

Viruses attached to host cell receptors may be able to act as a bridge for entry by other viruses. The sialic acid residues on the envelopes of vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV) can bind to the hemagglutinin of influenza virus present on the surface of influenza infected epithelial cells rendering the cells susceptible to infection by VSV or SFV through the apical and basolateral surface (Fuller et al., 1985) .

In some instances, structures on the plasma membrane which serve as viral receptors have specificity for a single virus, and in other instances, different viruses may share the same receptor. Based on these findings, Lonberg-Holm et al., (1976), introduced the concept of viral receptor families. In their

experiments, they were able to block the binding of one virus to the cell surface by preincubating the cells with an unrelated virus. Confirmation of the existence of "receptor families" will require biochemical determination of the specific binding sites.

The serotype specificity of some viruses can be correlated with differences in their receptors. For example, two serotypes of reovirus, types 1 and 3, differ in their ability to bind to primary cell cultures. Reovirus type 3 binds to neurons and lymphocytes whereas reovirus type 1 binds to ependymal cells (Weiner et al., 1980).

Estimates of the density of cell membrane receptor sites for certain viruses have been obtained using either growth assays or radiolabeled binding techniques. Despite the variety of different cell types and viruses analyzed, these estimates have been consistent, with values in the range of 10^4 to 10^5 sites per cell (Lonberg-Holm and Philipson, 1974; Birdwell and Strauss, 1974; McClintock et al., 1980; DeLarco and Todaro, 1976). Few studies have measured the affinity of viral binding to receptors in terms of the Kd of the equilibrium binding reaction. Dissociation constants are difficult to measure due to rapid penetration of the virus after binding. However, it has long been assumed that virus-cell interactions are of very high affinity, both because of the rapid time course of viral binding and the difficulty in disrupting bound virus by physical means (Lonberg-Holm et al., 1976).

The attachment of a virus to a cellular receptor site is only the first step in a series of events that ultimately results in viral replication. These include

internalization, uncoating, replication and assembly. Possession of a receptor is no guarantee that a cell can be infected. For example, Epstein-Barr virus attaches to a line of lymphoblastoid T cells (MOLT 4) but is not internalized (Menezes et al., 1977). This has also been shown to occur in non-virus systems. Low density lipoprotein normally binds to receptors on fibroblasts, but in individuals with defective cholesterol metabolism it binds but is not taken up (Brown and Goldstein, 1976). This indicates that the presence of a specific receptor on the cell surface is a necessary but not sufficient condition for viral replication. For non-permissive cells, it is important to determine whether restriction occurs at the receptor or at the intracellular level.

There are many factors which can determine the host range of a virus in addition to receptor interactions. Viruses may bind to cells but fail to penetrate the membrane barrier (Choppin and Scheid, 1980). Failure to penetrate the cell could in some cases be a receptor defect. However, abortive infections may also result from any stage of viral replication if the host cell machinery is not compatible with the synthesis and assembly of viral components.

Replication of some viruses can occur in a cell lacking appropriate viral receptors if the barrier to infection at the cell surface is circumvented. This has been achieved with poliovirus by two methods: 1. direct inoculation of viral nucleic acid into the cytoplasm of a cell lacking poliovirus receptors and 2. physical entrapment of the virus into the cell by fusion of the cellular membrane with Sendai virus (which incorporates polioviruses bound nonspecifically to the

cell surface) or by treatment of the cells with virus-containing liposomes (Enders et al., 1967; Wilson et al., 1977).

In some cases it is clear that other factors contribute to tissue tropism. For example, HIV can infect cells which lack the CD4 molecule (Clapham et al.,1989). In addition, HIV-1 does not infect mouse cells transfected with a CD4 cDNA, although it does bind to the surface of mouse cells bearing CD4, indicating that other factors in addition to CD4 are required for infection (Maddon et al., 1986; Levy, 1988). Other receptors are distributed widely among cell types or species making it difficult to understand how they alone can confer specificity. Poliovirus replicates in the gut and in motor neurons of primates. Its receptor has been characterized as a previously unidentified member of the immunoglobulin superfamily (Mendelsohn et al., 1989). However, mRNA encoding the receptor protein is expressed in a wide range of human tissue, including those that are not sites of poliovirus replication (Freistadt et al., 1990). Tissue tropism of poliovirus is therefore not governed solely by expression of the receptor in tissues, but may depend on tissue-specific modification of the receptor, additional factors required for poliovirus receptor function, or perhaps factors required for subsequent stages in virus replication.

It is unlikely that the structures on the cell surface which serve as viral receptors evolved merely for the purpose of virus binding. Viral receptors, most probably, serve other biological functions in a cell. It is known, for example, that bacteriophage receptors are components of the transport system for

low-molecular-weight sugars (Hazelbauer, 1975). Among animal virus receptors that have been clearly identified, the cellular functions of two of these receptors have been characterized. Intercellular adhesion molecule 1 (ICAM-1) is the major rhinovirus receptor (Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989). Lymphocyte function associated molecule 1 (LFA-1) mediates leukocyte adhesion by binding to ICAM-1 (Dustin, 1988). CD4, the receptor for the HIV virus, is a glycoprotein on the surface of T lymphocytes and some other cells that is believed to function as an adhesion molecule by binding to class II MHC molecules (Sattentau and Weiss, 1988).

Known virus receptors are listed in Table 1. The evidence is stronger for some of the receptors on this list than for others. Relatively few viral receptors have been identified unambiguously.

Two general classes of agents should, in theory, prevent virus entry into cells by blocking the attachment of virus particles to host cell receptors. The first group of agents resembles structurally the binding domain of the virus attachment protein. This moiety should competitively inhibit binding of the virus to the cellular receptor. Agents mimicking the virus binding domain are: antibodies directed against the binding site of the receptor; anti-idiotypic antibodies against antibodies to the binding domain of the virus attachment protein; natural ligands of the binding site of the receptor and synthetic ligands. For example, antibodies directed against the gp120-binding site on the HIV receptor CD4 block virus infection (Landau et al., 1988; Mizukami et al., 1988).

Table 1. Host cell receptors for DNA and RNA viruses. Adapted from Lentz, (1990).

Virus	Host cell receptor
DNA viruses	
Polyomavirus	Sialyloligosaccharides (Fried et al.,1981)
Simian virus 40	Class I MHC antigens (Attwood and Norkin, 1989)
Human adenovirus	Class I HLA MHC molecule (Chatterjee and Maizel,1984)
Human cytomegalovirus	Class I HLA MHC molecule via b 2-microglobulin (Grundy et al.,1987)
Epstein-Barr virus	C3d receptor CR2 of B lymphocyte (Fingeroth et al.,1984)
Vaccinia virus	Epidermal growth factor receptor (Eppstein et al.,1985)
Hepatitis B virus	Hepatocyte receptor for polymerized serum albumin via albumin (Machida et al.,1984) Hepatocyte receptor for polymeric IgA (Neurath et al.,1986) Sialoglycoprotein (Komai et al.,1988)
RNA viruses	
Poliovirus	Member of the immunoglobulin superfamily (Mendelsohn et al.,1989)
Human rhinovirus	Intercellular adhesion molecule-1 (ICAM-1) (Greve et al.,1989; Staunton et al.,1989; Tomassini et al.,1989)
Encephalomyocarditis virus	Sialoglycoproteins (Burness and Pardoe,1981)

Foot-and-mouth disease virus	Integrins (adhesion proteins) (Fox et al.,1989)
Reovirus 3	b Adrenergic receptor (Co et al.,1985) Sialoglycoproteins (Paul and Lee,1987), a-anomeric form of sialic acid (Paul, et al.,1989)
Simian and bovine rotavirus	Sialic acid (Fukudome et al.,1989)
Semliki Forest virus	Class I HLA and H-2 MHC molecules (Helenius et al.,1978)
Lactate dehydrogenase-elevating virus	Class II 1a MHC molecule of macrophage (Inada and Mims,1984)
Influenza virus	Sialyloligosaccharides (Paulson,1979; Paulson,1985)
Sendai virus	Sialyloligosaccharides (Paulson et al,1979)
Newcastle disease virus	Sialyloligosaccharides (Paulson,1979)
Vesicular stomatitis virus	Phosphatidylserine (Schlegel et al.,1983; Mastromarino et al.,1987) GM3 ganglioside (Mastromarino et al.,1987) Phosphatidylinositol (Mastromarino et al.,1987).
Rabies virus	Acetylcholine receptor (Lentz et al.,1982) Sialylated gangliosides (Superti et al.,1986)
Human T-cell leukemia virus (HTLV-1)	Class I HLA MHC molecule (Clarke et al.,1983) Interleukin 2 receptor (Lando et al.,1983; Kohtz et al.,1988)
Murine leukemia virus	Lymphoma cell surface IgM (McGrath et al.,1987) 622 amino acid, hydrophobic protein of unknown function (Albritton et al.,1989)

Radiation leukemia virus	T cell receptor-L3T4 molecule complex (O'Neill et al.,1987)
Human immunodeficiency virus-1	CD4 molecule (Dalgleish et al.,1984; Klatzmann et al.,1984b; Mc Dougal et al.,1986a) CD4 molecule interacting with class II HLA-DR MHC molecule (Mann et al.,1988)
Human immunodeficiency virus-2	CD4 molecule (Guyader,1987)
Simian immunodeficiency virus	CD4 molecule (Kannagi et al.,1985; Kornfeld et al.,1987; Hoxie et al.,1988)
Mouse hepatitis virus	100-120 kDa glycoprotein (Boyle et al.,1987)

A monoclonal antibody directed against the cellular receptor site for poliovirus types 1, 2 and 3, block infection of cells by all strains of the three poliovirus serotypes (Minor et al., 1984) and anti-receptor monoclonal antibody protects HeLa cells from infection by 78 of 88 human rhinovirus serotypes and from three coxsackievirus A serotypes (Colonno et al., 1986). Binding of reovirus type 3 to its receptor is blocked by an anti-idiotypic monoclonal antibody. This anti-idiotipic antibody is directed against a neutralizing antibody that recognizes the receptor binding domain of the reovirus hemagglutinin (Kauffman et al., 1983). Natural ligands have been shown in two instances to inhibit infection of cultured cells. The α-bungarotoxin prevents infection of cultured rat myotubes by rabies virus (Lentz et al.,1982; Tsiang et al., 1986) and epidermal growth factor prevents infection of murine L-cells by vaccinia virus (Eppstein et al., 1985). Synthetic peptides designed based on the binding domains of the viral attachment protein were effective in inhibiting binding of vaccinia virus (Eppstein et al.,1985), HIV (Pert et al., 1986), foot-and-mouth disease virus (Fox et al., 1989) and Epstein-Barr virus (Nemerow et al., 1989) to their cellular receptors. The second group of agents that should prevent attachment of virus to host cell receptors are agents resembling the binding domain on the receptor to which the virus attachment protein binds. This group of agents includes: antibodies directed against the binding domain of the virus; antibodies directed against antigenic sites surrounding the receptor binding site; anti-idiotypic antibodies directed against antibodies to the binding site on the receptor; the

receptor itself or fragments of the receptor molecule; and designed receptor mimics. Antibodies against the virus capsid or envelope have been shown to block infection. In same cases the antibodies recognize the viral protein involved in binding to the cellular receptor, in others the precise locations of their epitopes have not been determined. Synthetic peptides representing the binding domains of the virus have been successfully used as the immunogenic agents to obtain neutralizing antibodies in the case of hepatitis B virus (Itoh et al. 1986), foot-and-mouth disease virus (Bittle et al., 1982; Acharya et al., 1989; Fox et al.,1989) and human rhinovirus (McCray and Werner, 1987). Antibodies directed against regions surrounding the receptor-binding site block infection by rhinovirus (Rossmann et al., 1985) and influenza virus (Weis et al., 1988) by steric hindrance. Partial neutralization of HIV infection of T cells in vitro was accomplished with an anti-idiotypic that mimicks the CD4 molecule and binds to the HIV gp120 glycoprotein (Chanh et al.,1987). The examples of utilization of viral receptors to block infection are the use of the soluble, secreted forms of the CD4 molecule and fragments of CD4 to neutralize infectivity of HIV in vitro (Smith et al., 1987; Fisher et al., 1988; Hussey et al. 1988; Deen et al., 1988; Traunecker et al. 1988; Berger et al., 1988; Arthos et al., 1989; Chao et al., 1989). Synthetic peptides of CD4 residues 25 to 58 (Jameson et al., 1988) and a benzylated 83 to 94 peptide (Lifson et al., 1988) inhibit HIV-1-induced cell fusion, and certain glycoproteins inhibit hemagglutination of erythrocytes by influenza virus (Springer et al., 1969; Pritchett et al., 1987). A different

application of receptor mimics is to utilize them to target toxic agents to infected cells. The first 178 amino acids of CD4 linked to the active regions of Pseudomonas exotoxin A (Chaudhary et al., 1988) and soluble CD4 conjugated to the active subunit of the toxin ricin (Till et al., 1988) killed HIV infected cells.

Coronaviridae

General characteristics: The Coronaviridae is a family with one genus (coronavirus) comprising 11 viruses that infect vertebrates. Coronaviruses have been known for almost five decades. Schalk and Hawn, (1931) made the first description of a disease caused by coronaviruses. The responsible agent, avian infectious bronchitis virus (IBV), was first recovered in the laboratory in 1937 by Beaudette and Hudson. Another group of coronaviruses, murine hepatitis virus (MHV), was first recognized by Cheever et al., (1949) and independently by Gledhill and Andrewes, (1951). Doyle and Hutchings (1946) described transmissible gastroenteritis in swine (TGEV). These animal diseases were considered unrelated until after the human coronavirus genus (HCV) was defined (Tyrrell and Byone, 1965). IBV, MHV and the newly described human respiratory coronaviruses, were noted to have a similar appearance in negatively stained preparations (Almeida and Tyrrell, 1967; McIntosh, et al., 1967; Becker et al., 1967). These viruses displayed a characteristic fringe of large, distinctive, petal-shaped peplomers or spikes which resembles a crown. Their appearance was the base for the name coronaviruses (Tyrrell et al.,

1968). In addition to their morphological similarities, some of the human coronaviruses (HCV) were noted to be antigenically related to MHV (Tyrrell et al., 1968; McIntosh et al., 1969; Bradburne and Tyrrell, 1971).

The coronavirus group can now be defined by biological and molecular criteria. Coronavirus are round, moderately pleomorphic, large particles measuring 100-150 nm in diameter and are covered with club-shaped surface projections. The coronavirus particles have been observed to bud from the Golgi apparatus or endoplasmic reticulum and to accumulate in cytoplasmic vesicles (Becker et al., 1967). The coronavirus genome is a linear molecule of single-stranded RNA which is polyadenylated and infectious (Lomniczi, 1977; Schochetman et al., 1977; Wege et al., 1978; Brian et al., 1980). The genome RNA is 29,000 to 32,000 nucleotides long (Schochetman et al., 1977; Macnaughton and Madge 1977; Macnaughton et al., 1978; Stern and Kennedy 1980a,b; Yogo et al., 1977; Lai and Stohlman, 1978; Wege et al., 1981b,c ; Spaan et al., 1981; Leibowitz and Weiss, 1981; Weiss and Leibowitz, 1981; Garwes et al., 1975) and is capped (Lai and Stohlman, 1982; Lai et al., 1982; Weiss, 1983).

Virion polypeptides were studied by electron microscopy, radioisotopic labeling, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and tryptic peptide analysis (Sturman, 1981; Sturman and Holmes, 1977). Many molecules of a basic phosphoprotein, N (50 to 60K) encapsidate the plus-stranded genomic RNA to form a long, flexible nucleocapsid with helical

symmetry (Macnaughton et al., 1978; Stohlman and Lai, 1979; Sturman and Holmes, 1977). The lipid of the lipoprotein envelope is apparently derived from the rough endoplasmic reticulum or Golgi apparatus of infected cells (David-Ferreira and Manaker, 1965; Dubois-Dalcq, 1982; Massalski et al., 1982). The envelope consists of a lipid bilayer with two viral glycoproteins: M, the membrane associated glycoprotein and S, the spike glycoprotein (Garwes et al., 1976; Sturman and Holmes, 1977; Sturman et al., 1980) and in same cases a third glycoprotein HE, the hemagglutinin-esterase (Callebaut and Pensaert, 1980; King et al., 1985; Hogue and Brian, 1986). The M glycoprotein (20 to 30 K) is a transmembrane molecule that is deeply embedded in the envelope and only a small glycosylated amino-terminal region is exposed on the outer surface of the lipid bilayer (Rottier et al., 1984; Sturman and Holmes,1977). Addition of tunicamycin to cells after virus inoculation, which prevents the formation of N-glycosidic linkages to polypeptides, does not affect the synthesis or glycosylation of M (Holmes et al., 1981a,b; Niemann and Klenk,1981; Niemann et al.,1982). This suggested that M is O-linked glycosylated. Indirect immunofluorescence studies of MHV A-59 infected 17 Cl 1 cells showed that M is not transported to the cell membrane in the same manner as other viral glycoproteins. Budding of the virus from the Golgi apparatus may be the result of accumulation of M in this organelle (Holmes et al. 1981a,b). The second coronavirus glycoprotein, S (180 to 200K) is the structural protein of the peplomers that interact with the cell-surface receptors (Holmes et al., 1981a).

Most of the molecule lies outside the membrane, and is transported to the plasma membrane of infected cells (Cavanagh, 1984; Holmes et al., 1981a; Sturman, 1981). Antibodies to S can neutralize viral infectivity (Collins et al., 1982; Fleming et al., 1983). Cleavage of S to two 90K polypeptides by host-cell proteases can activate cell-fusing activity (Holmes et al., 1984; Sturman and Holmes, 1984). A third membrane glycoprotein, referred to as hemagglutinin (HE) can be found in some coronaviruses. HE is a dimer of molecular weight 130 to 140 K and its presence permits virions to bind to cell membrane molecules containing 9-O-acetylated sialic acid (Callebaut and Pensaert ,1980; King et al. 1985; Hogue and Brian, 1986; Vlasak et al., 1988).

Table 2 shows the coronaviruses their natural hosts, and the major diseases that they induce. There are at least four distinct antigenic groups of coronaviruses, based on serologic studies with immunofluorescence assays, virus-neutralization tests, enzyme-linked immunoassays, and immunoelectron microscopy (Pedersen et al., 1978; Siddell et al., 1983; Sturman and Holmes, 1983; Wege et al., 1982). Viruses within each group show partial antigenic cross-reactivity, but they are readily distinguishable by their host specificity and clinical syndromes.

Analysis of murine coronaviruses indicates that there is considerable diversity between different strains. Wege et al., (1981b,c) and Lai and Stohlman, (1981) found that the MHV-JHM strain differed significantly from MHV-1,-2,-3, and -A59 strains based on oligonucleotide fingerprint patterns

Table 2. Coronaviruses: names and diseases caused by this genus. Others refers to infectious peritonitis, pancreatitis, runting, nephritis and adenitis. Adapted from Holmes, (1989).

Antigenic group	Virus	Diseases
I	Human corona virus-229E	Respiratory infection
	Porcine transmissible gastroenteritis virus	Respiratory and enteric infection and others
	Canine coronavirus	Enteric infection
	Feline enteric coronavirus	Enteric infection
	Feline infectious peritonitis virus	Respiratory, enteric, neurologic infection, hepatitis and others
II	Human respiratory coronavirus OC-43	Respiratory infection
	Mouse hepatitis virus	Respiratory, enteric, neurologic infection and hepatitis
	Porcine hemagglutinating encephalomyelitis virus	Respiratory, enteric and neurologic infection
	Bovine coronavirus	Enteric infection
	Rabbit coronavirus	Enteric infection and others
	Sialodacryadenitis virus	Parotitis and others
III	Avian bronchitis virus	Respiratory infection and other
IV	Turkey coronavirus	Turkey bluecomb disease. Respiratory and Enteric infection
unclassified	Human enteric coronavirus	Enteric infection ?

and that MHV-3 and MHV-A59 were the closest strains. Lai and Stohlman, (1981) attempted to correlate strain-specific oligonucleotide differences with hepatotropism and neurotropism of MHV-3 and -A59 and found two genetic regions that differ between the strains. The genetic relatedness of coronaviruses has also been analyzed by nucleic acid hybridization. Using a cDNA probe representative of the entire genome of MHV-A59, Weiss and Leibowitz, (1981) found that MHV-3 and -A59 were more closely related to one another than either was to MHV -JHM. The human coronavirus HCV-229E appeared to be quite unrelated to MHV when this technique was applied.

Role of receptors in coronavirus species specificity : Coronavirus are highly species specific. They usually infect only one species or a few closely related species. Mouse hepatitis virus, for example, causes natural infection in mice, and infects rats only by abnormal (e.g., intracerebral) routes, at a very young age and with a highly neurotropic virus strain (Hirano et al., 1980; Sorensen et al., 1980; Wege et al., 1981a; Sorensen and Dales, 1985; Watanabe et al., 1987). Coronaviruses also exhibit a high level of species specificity in cell culture, infecting only cells derived from their normal host and occasionally from species that are susceptible to antigenically related coronaviruses (Manaker et al., 1961; King and Brian, 1982; Schmidt et al., 1979; Hogue et al., 1984; Binn et al., 1980; Evermann et al., 1981; Barlough et al., 1983).

Solid phase assays were performed to determine the species specificity of receptor recognition of antigenically related coronaviruses (Compton, 1988).

Unlike for MHV, there may be cross-reactivity at the level of virus receptor interaction for canine coronavirus (CCV), feline infectious peritonitis virus (FIPV), human coronavirus HCV-229E, and transmissible gastroenteritis virus of swine (TGEV). Species specificity for the coronaviruses of these animals may be determined at least in part by a factor or factors subsequent to the initial virus attachment phase.

Although most coronaviruses do not cause hemagglutination, several coronaviruses including bovine coronavirus (BCV), hemagglutinating encephalomyelitis virus of swine (HEV), human respiratory coronavirus OC43, and some strains of infectious bronchitis virus (IBV) can cause hemagglutination. These virions and certain strains of MHV (MHV-DVIM and MHV-JHM) contain an additional membrane glycoprotein, called HE which has hemagglutinin and acetyl esterase activities (Vlasak et al., 1988; Luytjes et al., 1988; Sugiyama et al., 1986; Taguchi et al., 1985). Coronavirus HE has sequence homology to the glycoprotein of influenza C (Luytjes et al., 1988). It is possible that the interaction of the viral HE glycoprotein with a carbohydrate on the cell surface can serve as an alternate way for some coronavirus to infect cells in vitro or in vivo. On the other hand, the hemagglutinin of some coronaviruses may, like that of polyomavirus, be irrelevant in the infectious process. Several lines of evidence show that MHV-A59 does not bind to susceptible cells by recognizing 9-O-acetylated neuraminic acid containing receptors. First, MHV-A59 does not express the HE glycoprotein. The HE gene

of MHV-A59 lacks an initiator methionine, and cells infected with MHV-A59 do not make an RNA which would be translated to make the HE glycoprotein (Luytjes et al., 1988). Second, MHV-A59 binds to only a single glycoprotein in virus overlay protein blot assays (Boyle et al., 1987). Third, neuraminidase treatment of murine cells, which should remove 9-O-acetylated neuraminic acid residues, fails to prevent binding of MHV-A59. Finally, pretreatment of cells with an anti-receptor monoclonal antibody blocks virus binding and prevents virus infection (K.V. Holmes, personal communication).

Many experimental approaches were used to determine whether the species specificity of MHV-A59 infection is the consequence of the S glycoprotein binding to a domain of a glycoprotein which is only expressed on murine tissues. Immunofluorescence experiments on cell lines showed that the anti-MHV receptor antibody bound to MHV-susceptible murine cell lines and not to cell lines from human, hamsters, cats and dogs. MHV-A59 binds only to brush border membranes of BALB/c mice, and not to brush border membranes from human, pig, cat, dog, rat and cow (Compton, 1988). These observations strongly support the role of receptors in determining the species specificity of MHV. The interaction of the MHV receptor glycoprotein with the S glycoprotein of MHV-A59 virions appears to be sufficient for initiation of virus infections.

Features of the MHV receptor : MHV is a common murine pathogen and is extremely contagious (Parker, 1980; Lindsey, 1986). Many serologically distinguishable strains of MHV cause inapparent enteric infection, infantile

diarrhea, hepatitis, respiratory infection, and acute and chronic demyelinating neurological diseases (Hirano et al.,1975, Wege et al., 1981a; Talbot and Buchmeier, 1985). Approximately 25 different strains or isolates of MHV have been described. Of that number, six have been studied most extensively and are generally considered the prototype strains : MHV-1, MHV-2, MHV-3, MHV-JHM (MHV-4), MHV- S, and MHV- A59.

One of the classic examples of host resistance to virus infection is mouse hepatitis virus (MHV). Mouse strains differ markedly in their susceptibility to MHV. Peritoneal macrophages isolated from the different mouse strains showed susceptibility or resistance to MHV corresponding to that of the intact animal (Bang and Warwick, 1960; Stohlman et al., 1980; Dupuy et al., 1984; Knobler et al., 1984; Dindzans et al., 1986). Mice fully susceptible to MHV, such as BALB/c and C57BL/6, are killed by infection with a small number of virions. Large amounts of virus can be isolated from target organs such as liver, and extensive lesions are detected by histopathology (Barthold, 1986). SJL/J mice are resistant to infection even with large doses of virus, develop no histologic lesions, and do not replicate virus in target tissues (Stohlman and Frelinger, 1978). There are two mice strains that are semi-resistant to infection. The semi-resistant A/J strain can be infected by small doses of MHV-3, and large amounts of virus can be isolated from target tissues, but there is little or no histopathology and the infected animals survive (Dindzans et al., 1986). The semi-resistant C3H strain can be infected by MHV, but develops only focal

lesions and produces only moderate yields of virus in the liver (Bang and Warwick, 1960; Woyciechowska et al., 1984). Resistance to MHV-A59 replication in macrophages is controlled by a single locus expressed in a recessive fashion (Smith et al., 1984). The locus is refered to as hv-2 and in near the centromere of chromosome 7. The mechanism by which the locus confers resistance is unclear, but it appears to control an event preceding virus-specific RNA synthesis (Smith et al., 1984). The semi-resistance of the C3H mouse also is encoded by a single autosomal recessive allele which has not been mapped, although congenic semi-resistant strains of mice on a Pri background have been developed (Weiser et al., 1976). Inheritance of semi-resistance of A/J mice is more complex, and appears to be due to at least 2 non H2-linked autosomal recessive genes, one of which may be monocyte procoagulant activity (Dindzans et al., 1986). Several hypotheses have been proposed to explain the mechanism of host resistance to MHV. Resistance could be due to lack of a receptor for the virus on target cells, defective replication of viral RNA or virion assembly in cells from resistant animals, altered host immune response to MHV, or abnormal interferon induction by MHV. In an attempt to clarify the mechanism of resistance to MHV, binding of MHV-A59 to plasma membranes of the two most prominent target tissues in genetically susceptible BALB/c mice was studied by (Boyle et al., 1987). A solid phase virus receptor assay was developed to detect binding of MHV-A59 to enterocytes and hepatocytes of susceptible and resistant mice. Brush border

membranes isolated from the small intestine of BALB/c and SJL/J mice were immobilized on nitrocellulose in a dot blot apparatus, and then incubated with MHV-A59 virus. Virus bound to the intestinal brush border membranes was detected immunologically with antibody directed against the peplomer glycoprotein S and radioiodinated staphylococcal protein A. Virus binding to brush border membranes from BALB/c mice was directly proportional to the amount of membranes used, but no binding of virus was detected to brush border membranes from SJL/J mice. Similar findings were made with hepatocyte membranes from BALB/c and SJL/J mice (Boyle et al., 1987). The results of this solid-phase assay are in accordance with a previous observation that radiolabeled MHV virions bound to the plasma membranes of splenocytes from BALB/c but not SJL/J mice (Holmes et al., 1986). These data suggested SJL/J mice are resistant to MHV infection because they fail to express a specific receptor for MHV on the membranes of the normal target cells for the virus, express an altered molecule that lacks MHV-binding activity, or express too little receptor to be detectable. The availability of a receptor-deficient mouse strain, SJL/J, served as an important tool for subsequent studies on the interaction of the S glycoprotein of MHV with receptors on intestine and liver cell membranes from the susceptible BALB/c mice.

MHV binding activity is not destroyed by treatment of membranes by SDS. Determination of the molecular weight of the receptor on BALB/c membranes was accomplished by Boyle et al. (Boyle et al., 1987), in a virus

overlay protein blot assay (VOPBA). Membrane proteins from BALB/c or SJL/J intestinal brush borders or hepatocytes were solubilized in SDS, separated by SDS-PAGE, and blotted onto nitrocellulose sheets. The sheets were then incubated sequentially with bovine serum albumin to block non-specific binding, MHV, anti-E2 antibody, and radioiodinated staphylococcal protein A. A large number of membrane proteins was detected by Commassie blue staining of the gels. With BALB/c brush border membranes or hepatocyte membranes, MHV bound only to a single broad band with a molecular weight of approximately 100 to 110 kDa. None of the membrane proteins of cells from SJL/J mice showed any virus-binding activity. The virus binding activity of enterocyte membranes was destroyed by treatment of the membranes with trypsin and other proteases (Boyle et al., 1987). Treatment of membranes with neuraminidase or endoglycosidase F before incubation with virus did not prevent virus attachment. Two-dimensional isoelectric focussing and SDS-PAGE showed that the MHV receptor has a pI of 3.5. The 110 kDa receptor was shown to be a glycoprotein by quantitatively recovering it from solubilized membranes with lectin affinity chromatography (Holmes et al., 1987).

Polyclonal antibodies directed against the MHV receptor were prepared in Dr K. V. Holmes' laboratory by immunizing receptor-deficient SJL/J mice with intestinal brush border membranes of receptor-positive BALB/c mice. This antibody recognized only a 100 to 110 kDa protein in immunoblots of BALB/c

brush border membranes. Hybridomas were prepared from spleen of these animals. One of the hybridomas (MAb CC1) produces a monoclonal antibody (MAb) directed against the receptor . MAb CC1 blocks infection of mouse fibroblasts in vitro with MHV-A59 when virus was incubated with cells that has been pretreated with the antibody. The MAb CC1 also blocks infection of mouse fibroblasts with other strains of MHV, MHV-JHM, MHV-3, MHV-S and MHV-1. (K.V.Holmes, personal communication). This indicates that on the mouse fibroblast cell lines, the 110 kDa glycoprotein receptor is the only receptor for all 5 of these prototype strains of MHV. These observations also suggest that MAb CC1 binds to the native receptor glycoprotein at a site which is near enough to prevent virus binding by steric hindrance. Fluorescent antibody labeling of murine intestine showed that MAb CC1 specifically recognized the apical brush border of intestinal epithelial cells of BALB/c mice but failed to react with membranes of SJL/J intestine (Holmes et al.,1989). The relative levels of expression of the MHV receptor glycoprotein on different tissues of MHV-susceptible BALB/c mice were determined by binding of radioiodinated MAb CC1 to membranes prepared from these tissues. Expression of the MHV receptor glycoprotein was greatest in the large and small intestines and liver of BALB/c mice (William et al., submitted for publication). These three tissues are the major natural target organs for MHV.

MAbCC1 was used for affinity purification of the receptor glycoprotein from livers of MHV-susceptible Swiss Webster mice (Williams et al., 1990). The

MHV receptor and an antigenically related protein of 48 to 58 kDa were copurified and then separated by preparative SDS-PAGE electrophoresis. The first 15 amino acids of the receptor were determined, and a synthetic peptide of this amino acid sequence was prepared. Rabbit antiserum made against this peptide bound to the MHV receptor glycoprotein and the 48 to 58 kDa protein from livers of MHV-susceptible BALB/c mice and Swiss Webster mice and from the intestinal brush border of BALB/c mice (Williams et al., 1990). The 48 to 58 kDA glycoprotein appears to be closely related to the MHV receptor, since they share both the amino-terminal antigenic domain and the MAb CC1-binding domain. However, unlike the MHV receptor glycoprotein, the 48 to 58 kDa protein binds MHV-A59 virions poorly compared with its binding to MAb CC1 (Williams et al., 1990). These observations indicate that the domain of the receptor that binds the S glycoprotein of MHV-A59 is not identical to the domain which binds MAb CC1. These two domains must be close enough to permit blocking of the virus by the antibody. The antibody against the first 15 amino acids of the MHV receptor was tested in immunoblots of intestinal brush border membrane and hepatocyte membranes of the MHV-resistant SJL/J mice (Williams et al., 1990). This antibody recognized two slightly smaller proteins in the two tissues examined. These proteins are 5 to 10 kDa smaller than the MHV receptor and the 48 to 58 kDa related protein from Swiss Webster or BALB/c mice. The absence of the virus-binding site on the receptor-homologous proteins from SJL/J mouse liver and intestine may play a role in determining the

resistance of SJL/J mice to MHV-A59. The differences in molecular mass, virus binding, and MAb CC1 binding activities found between the BALB/c mice and SJL/J related proteins may be the result of different phenomena. These could include a difference in post-transcriptional processing, deletion or mutations in the virus binding and MAb CC1 binding domains, and alternative splicing in liver and intestinal epithelial cells of SJL/J mice.

The purpose of my work was to clone and characterize the cellular receptor for the mouse hepatitis virus.

MATERIALS AND METHODS

Materials : All radioactive compounds used, α [^{32}P] UTP, α [^{32}P] dCTP, γ [^{32}P] ATP, [^{35}S] - methionine and α [^{35}S] dATP were obtained from New England Nuclear. All isotopes were used in specific labelling procedures as described in detail in the methods section.

Enzymes used in the experiments were from several sources. Amplitaq, the cloned thermostable DNA polymerase from Thermus aquaticus was purchased exclusively from Perkin Elmer Cetus. For DNA sequencing, the Sequenase kit was obtained from US Biochemicals and the Sequagel sequencing system was purchased from National Diagnostics. Moloney murine leukemia virus reverse transcriptase (MMLV) , MMLV RNase H- reverse transcriptase, T4 DNA polymerase, T4 polynucleotide kinase, terminal

deoxynucleotidil transferase, T4 DNA ligase, T7 RNA polymerase, nick translation kit, and all the restriction enzymes used (EcoR V, Sal I, EcoR I, Hinc II, Sst I, Sst II, BamH I, Mbo I, Xho II, Bgl II) with the exception of Not I were purchased from Life Technologies. All these enzymes were used according to the manufacturer's protocols as adapted and described in detail in the Methods section. The reticulocyte translation system and the Not I restriction enzyme were obtained from Promega. All the agarose gels were made at the appropriate percentage using SeaKem GTG agarose.

The oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer (Applied Biosystems,Inc.) at USUHS and checked for purity by polynucleotide kinase labelling and sequencing gel analysis. Oligonucleotides were typically 95% pure. The sequence of the oligonucleotides employed is shown in Table 3.

The vector pUC18 was purchased from Life Technologies and BlueScript SK+ from Stratagene. Subcloning efficiency and library efficiency competent cells were obtained from Life Technologies. The nytran and nitrocellulose membranes employed for Northern, Southern and Western blot analysis and filters for library and colony screening were obtained from Schleicher and Schuell. The Centricon 100 microconcentrators were purchased from Amicon.

Specialty reagents that were not used routinely are described in the Methods section.

RNA and DNA preparation : BALB/c and SJL/J mice were kept without food

Table 3 . Sequence of the oligonucleotides employed. The oligonucleotides are listed in 5' to 3' orientation. Bases in parentesis designate positions of degeneracy within the oligonucleotide.

1. GA(GA)GTGACCATTGA(GA)GCTGTGCCCCCCCA(GA)GTGGC

2. CCATGGAGAAGGCTGGGG

3. CAAAGTTGTCATGGATGACC

4. CTAAGCAGTTGGTGGTGCA

5. GCATGCGCGCGGCCGCGGATT$_{(18)}$X

6. GCATGCGCGCGGCCGCGG

7. GA(GA)GT(GA)AC(CT)AT(CT)GA(GA)GC(GA)GT

8. GA(GA)GT(GA)AC(CT)AT(CT)GA(GA)GC(CT)GT

9. CAGGTGGC(CT)GA(GA)GA(CT)AA(CT)AA(CT)G

10. CGGCAGAGAGATAATATACAG

11. GAGGTTGAGGTTTGACCCTGG

12. GTAGACTCCCATATCCTTCATGG

13. GGTCGACTGGGGCTTCTCATTGATAAG

14. ATCACTACTAATAATAGCGG

15. GGTCGACAGATTGTGAACAAGTAGAAGAAC

16. GTTGTCTTCAGCAACCTGGG

17. ACGGTACCATGGCCCA(CT)CTGTCTGC(CT)CC

18. AAGTCACGTCATGAGTCCGACAG

19. GATCCTGTGAT

20. AAGTCACGTCATGAGTCC

21. CTTGTCGAATGACATTGGA

22. GAAATCTCGAATCCAGTCAG

23. ACAGTCAAGAACATTACAGT

supply overnight to reduce the quantity of liver glycogen. Animals under anesthesia were sacrificed by cervical dislocation, opened in an aseptic manner and for DNA preparation, the livers were removed and snap frozen in liquid nitrogen. For preparation of RNA the livers, small intestines, and large intestines were washed with ice cold phosphate buffer saline (PBS) and the intestines were carefully dissected and flushed.

Genomic DNA was prepared by the method of Blin and Stafford, (1976) and modified as follows. Frozen liver was powdered in liquid nitrogen using a mortar and pestle. The tissue was then mixed with ten volumes of LET buffer (50 mM Tris.Cl pH 8.0, 50 mM NaCl, 0.1 M EDTA and 0.05 % SDS) and 100 µg/µl of proteinase K (Life Technologies) was added. The solution was gently rocked at 50°C for 5 hours, additional proteinase K (50 µg/ µl) was added and the digestion was continued overnight. Once the solution was clear, the sample was incubated with 50 µg/ml of RNase A. RNase A was treated by boiling in 0.1M sodium acetate pH 5.0 to inactivate residual DNase activity. The DNA was phenol:chloroform extracted twice, followed by one chloroform extraction. A wide bore pipette was used to recover the DNA and prevent DNA strand breakage. The DNA was spooled by the method of Marmur, (1961) and rehydrated in TE (10 mM Tris.Cl pH 8.0, 1 mM EDTA). This preparation of DNA was checked by electrophoresis on a 0.8% agarose gel and migrated at a size greater than 50 kilobases.

Total tissue and cellular RNA was prepared by the method of Chomczynski and Sacchi, (1987). This procedure was designed to yield quality RNA for Northern blots and it was modified as described below to produce RNA for RNAPCR. Briefly, tissue samples or pellets of tissue culture cells were homogenized in a 20-fold excess volume of 4M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1 M β-mercaptoethanol. After homogenization 0.1 volumes of 2 M sodium acetate pH 4.0, 1 volume of water saturated phenol and 0.2 volumes of chloroform:isoamylalcohol (49:1) were added and cooled on ice for 15 min. This causes a phase separation with the RNA remaining in the aqueous phase and the DNA partitioning to the organic phase. Following centrifugation at 10,000xg for 20 minutes the aqueous phase was recovered by centrifugation and the RNA was precipitated with an equal volume of isopropyl alcohol. The RNA containing pellet was washed twice with 70% ethanol and dissolved in RNase free H_20. The water was made RNase free by filtering twice with 0.22 micron Milllex-GS filter units. The RNA was then reprecipitated with 0.1 volumes of 3 M sodium acetate pH 5.0 and 2.5 volumes of ethanol in 1.5 ml eppendorf centrifuge tubes. Stocks of RNA were stored at this point at -70°C and not processed further until needed. Prior to use in RNAPCR the sodium acetate-ethanol precipitated RNA was recovered by centrifugation at 10,000xg for 15 minutes. After washing with 70% ethanol the RNA pellet was dissolved in RNase free H_2O and precipitated for 3 hours on ice by the addition of LiCl to a final concentration of 2.5 molar. The LiCl precipitated

RNA was recovered by centrifugation at 10,000xg and the RNA pellet was washed twice with 70% ethanol. The RNA was dissolved in RNase free water and quantified by UV spectroscopy.

Poly A+ RNA was prepared with the polyATtract mRNA isolation system (Promega) or the Fast Tract kit (Invitrogen) according to manufacturer's protocols.

Preparation of plasmid DNA : Primary transformants of DH5 α were verified to carry recombinant plasmids by colony hybridization and analysis of small scale preparations of plasmid DNA (minipreps). Colony hybridization was performed by the method of Grunstein and Hogness, (1975) using labeled oligonucleotides as probes. Minipreps were performed by the lysozyme alkaline lysis method of Ish-Horowicz and Burke, (1981). This method of small scale plasmid preparation usually yielded enough DNA for restriction enzyme digestion and preliminary DNA sequence determination. Once the plasmid was completely verified, the E. coli containing the recombinant plasmid was grown in 1 liter cultures of super broth (0.089 M $KHPO_4$ pH 7.5, bacto-yeast extract 24 g, bacto-tryptone 12 g, glycerol 4 ml) with ampicillin (Sigma) at a concentration of 50 μg/ml. Double cesium chloride (Life Technologies) density gradient banded plasmid DNA was prepared by the detergent lysis method of Clewell and Helinski, (1972).

Subcloning of PCR products : Amplified material was phenol:chloroform

extracted and passed through a Centricon 100 microconcentrator, or electrophoresed on acrylamide or agarose gels. Volumes of 35 to 55 µl were recovered after passage of the samples in 2 ml of TE through a Centricon 100 for 1 - 2 hours at 2,800 rpm. Fragments bigger than 500 bp were recovered from agarose gels by incubation with glass milk beads under the recommendations in the geneclean kit (Bio 101). Smaller products were run on polyacrylamide gels and recovered using the soak and crash method. This method consists of excision of the visualized band and elution of the DNA after incubation for 12 hours in 0.5 M ammonium acetate, 10 mM magnesium aceteate, 1 mM EDTA pH 8.0 and 0.1 % SDS. The sample is then centrifuged at 12,000xg at 4°C and further purified in a Centrex column (Schleicher and Schuell). Elimination of unincorporated nucleotides and primers is necessary because these interfere with future enzymatic reactions. Denny and Weissman, (1990) described that Taq polymerase adds an extra adenosine at the 3' end of the newly synthesized product. Elimination of the extra base is necessary before performing blunt-end ligation to the plasmid. This was accomplished by a 3 min incubation of the purified product with 1 unit of T4 DNA polymerase without added nucleotides followed by the addition of 2.5 mM dNTPs for 13 min. After phenol:chloroform extraction and precipitation in ethanol the product was incubated with the plasmid that had been digested for blunt ligation with Hinc II or EcoR V in a 10-20 µl reaction in the presence of T4 DNA ligase, for 12 hours at 4°C. When

one of the primers had a restriction enzyme site at its 5' end, after treatment with T4 DNA polymerase, the sample was incubated at 37°C for 2 hours with the appropriate restriction enzyme and phenol:chloroform extracted before ligation to the digested plasmid. Since the primers do not have a 5' phosphate group, the plasmids could not be treated with phosphatase to avoid re-circularization of empty vector. This was minimized by agarose gel electrophoresis of the plasmids after digestion with the appropriate restriction enzymes and purification from the agarose with the geneclean kit (Bio 101). Because of the inefficiency in the subcloning of PCR products, several reactions were pooled for ligation to 100 ng of plasmid DNA. A portion of the ligation reactions was used to transform DH5 α competent cells and spread on isopropylthio -β-D-galactosidase (IPTG)(12.5 mg/l), 5-Bromo-4-chloro-3-indolyl-β- D-galactosidase (X-gal) (0.0025 %), ampicillin (25 μg/ml) LB-plates. The vectors used for subcloning permit color selection of recombinant plasmids by complementation of the α portion of the β galactosidase gene. The plates were incubated at 37°C overnight and white or light blue colonies were plated in duplicate for colony screening as described above.

RNAPCR : RNAPCR was performed as two separate steps. The first was the synthesis of cDNA directed by a sequence specific oligonucleotide primer. Since the cDNA synthesis was directed by different oligonucleotide antisense primers for each RNA preparation, the details of each RNAPCR are specified in

the Results section and the nucleotide sequence of the primers is listed in Table 3 of this section.

As a general method, 0.5 - 4.0 µg of either poly A+ RNA or total RNA was mixed with 100 pmoles of antisense primer in a final volume of 10.5 µl. This mixture was heated to 70°C for 5 minutes, then cooled on ice. To this preparation 5X reverse transcriptase buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 0.1 M DTT, 1.5 µl of 10 mM each dNTP were added and after vortexing stored on ice for 5 min. The volumes added were adjusted to the final concentration indicated by the manufacturer. The reverse transcriptase reactions were initiated by the addition of 500 units of MMLV reverse transcriptase or MMLV-RNase H- reverse transcriptase. The RNase H- form was used when cDNAs of longer than 700 bp were synthesized. The reverse transcriptase reaction was incubated at 37°C for 30 min to 1 hour. The reaction was terminated and the cDNA-RNA duplex denatured by boiling the reaction for 10 minutes. The polymerase chain reaction (PCR) portion of this two part protocol was initiated by the addition of 75 µl of a solution converting the reverse transcriptase reaction conditions to PCR reaction conditions. The solution components were as follows: 9 µl of 10X Taq buffer (500 mM KCl,100 mM Tris.Cl pH 8.45, 15 mM $MgCl_2$, 0.1% gelatin), 6.5 µl of 10 mM each dNTP, 150 pmoles of sense primer, 125 pmoles of antisense primer and H_20. Two and

a half units of Amplitaq was added individually to each tube, the reactions were overlaid with 100 µl of mineral oil (Sigma) and subjected to 25-30 cycles in a Perkin Elmer Cetus thermal cycler. Cycling conditions are described below.

DNAPCR : With DNAPCR, 10^3 -10^4 copies of target DNA were completely denatured by boiling for 10 min followed by rapid cooling on ice. No other factor had a greater impact on the success or failure of PCR than the denaturation of the target template. Once denatured the template was mixed with Taq buffer, 150 pmoles of sense and antisense primers, 7 µl of 10 mM each dNTPs and 2.5 units of AmpliTaq and overlaid with mineral oil.

Phage DNA from isolated plaques was amplified by elution of the plaque in 10 µl of H_20 for 30 min at room temperature. This starting material was boiled for 10 min and the nucleotides, Taq buffer and sense and antisense primers at the concentrations described above were added to a final volume of 20 µl. After addition of 2.5 units of AmpliTaq the samples were overlaid with mineral oil and cycled in the machine.

For PCR amplification of plasmids, the plasmid was linearized with a restriction enzyme that would not cut the DNA in the area between the primer sequences and diluted to 100 pg in H_2O containing 123 bp ladder (Life Technologies) at a concentration of 1 ng/ml. The linearized plasmid DNA was then denatured by boiling and served as template for the PCR.

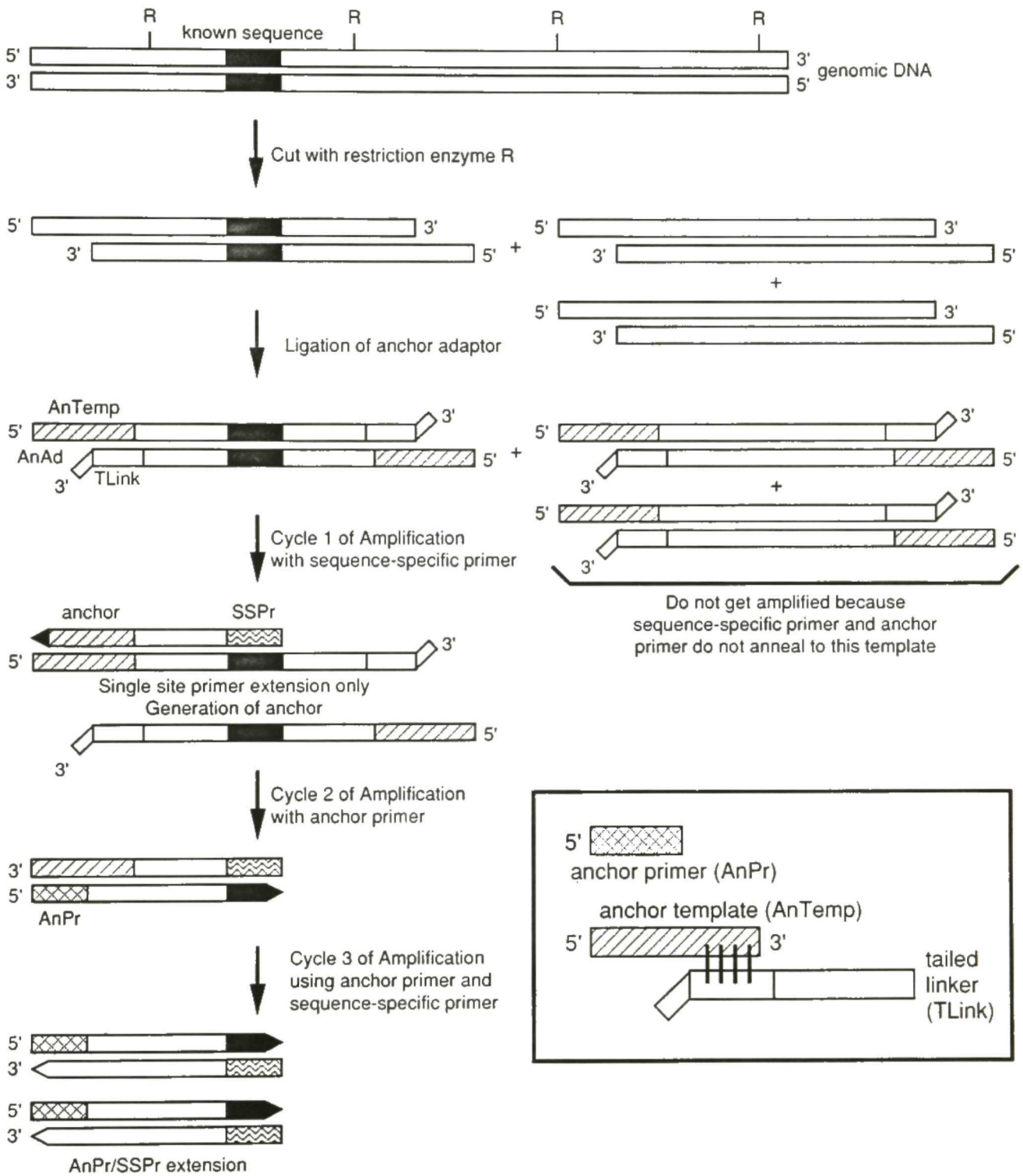
Single site PCR amplification of dsDNA : The basic protocol used is shown in

figure 1 and is based on the report of Roux and Dhanarajan, (1990). Liver DNA was heated at 60°C for 15 min followed by digestion of 10 µg of DNA with the enzymes BamH I, Bgl II, Xho II and Mbo I in four separate reactions in the appropriate buffer conditions for 3 hours. A 1:1 molar ratio mixture of kinase-treated oligonucleotide 19 (1µg) and oligonucleotide 18 (2 µg) were mixed with 4 µg of the digested DNA in a total volume of 38 µl. The mixture was heated to 65°C for 10 min and allowed to hybridize by slow cooling at room temperature for 2 hours. Ligation of the hybridized components was accomplished by addition of T4 DNA ligase and T4 DNA ligase buffer (10 X buffer: 30 mM Tris.Cl pH 7.8, 100 mM Mg Cl_2, 100 mM DTT, 5 mM ATP) at 4°C for 12 hours. The mixture was phenol:chloroform extracted and ethanol precipitated. The pellet was dissolved in 1.5 ml of dH_2O and passed through a Centricon 100 filter to remove unligated oligonucleotides. A recovery of the adaptor-ligated DNA of 100% was assumed and 0.5 µg were mixed with 150 pmoles of primers 20 and 15 and the material was amplified using the standard conditions for DNAPCR of long products. One microliter of the amplification reactions was re-amplified with primers 20 and 16 for another 25 cycles under the same amplification conditions. The sequence of the primers is listed in Table 3.

Amplification of the 5' end of the receptor mRNA with one sequence specific primer : Five micrograms of total RNA were reverse transcribed with primer 15

Figure 1. Single site PCR amplification of dsDNA. Scheme of the basic protocol employed for the amplification of the 5' end of the MHV receptor using restriction enzyme digested genomic DNA as starting material. The relative positions of the anchor primer, the anchor template and the tailed linker containing a 3' end noncomplementary sequence to inhibit primer extension is shown in the box.

One-Sided PCR Amplification of dsDNA



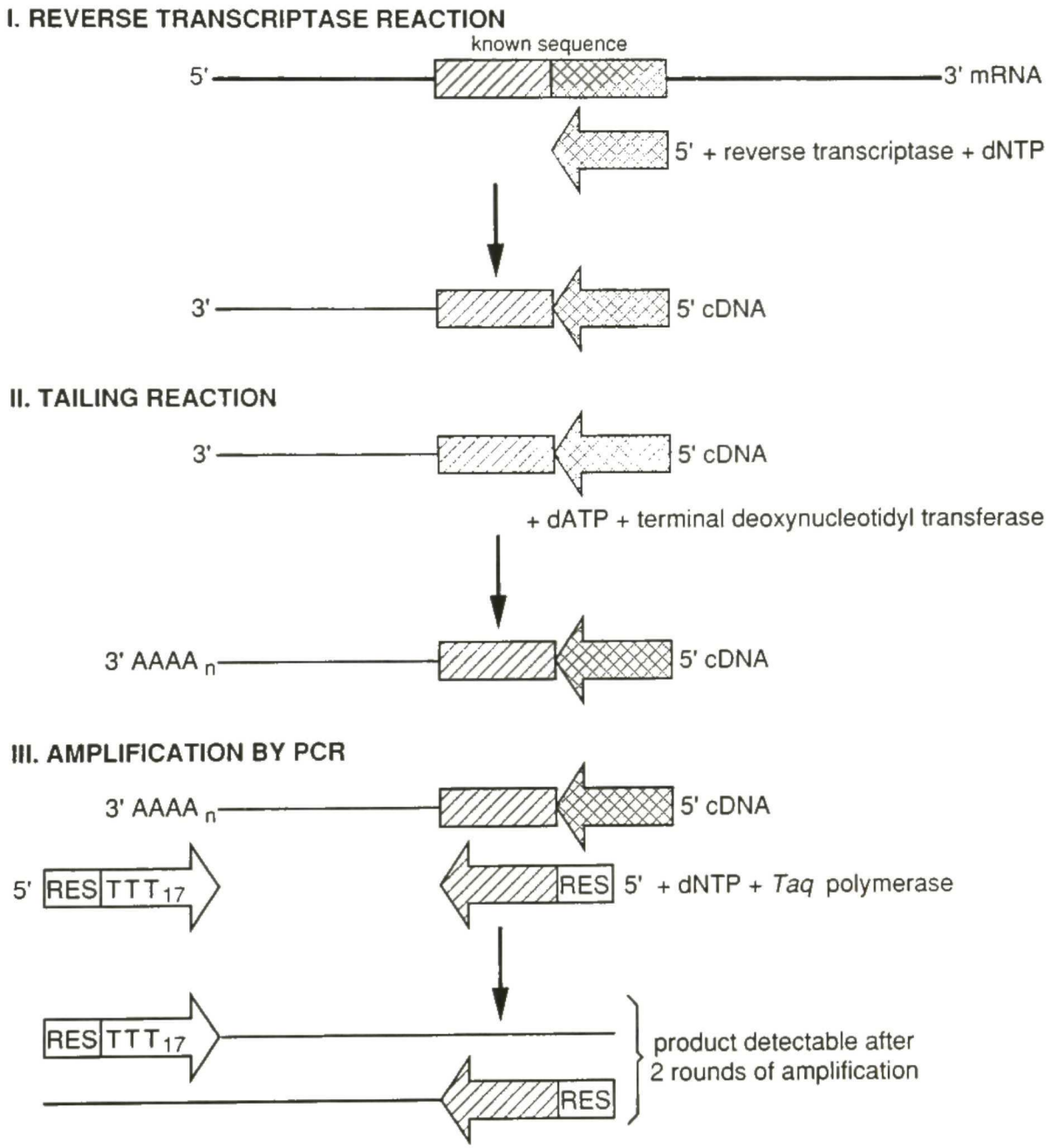
as described above for the RNAPCR. After the incubation at 37° C, the reaction was phenol:chloroform extracted, the aqueous phase extracted with chloroform and ethanol precipitated overnight at 4 °C with the addition of 1 µl of glycogen (Behringer Manheim) as a carrier and 0.1 volumes of sodium acetate pH 5.0. After centrifugation the precipitate was washed with 70% ethanol and resuspended in dH_20 for tailing the 5' end of the cDNA with terminal deoxynucleotidyl transferase (TdT). The cDNA was incubated at 37° C with 1 mM dATP, TdT buffer (5X buffer: 500 mM cacodylate, pH 6.8, 5 mM $CoCl_2$, 0.5l mM DTT, 500 µg/ml BSA) and 15 units of TdT in a 30 µl reaction. After 1 hour,l the reaction was phenol:chloroform extracted, and precipitated as described above with addition of glycogen. Amplification of the tailed cDNA was performed under standard conditions using 150 pmoles of primer 15 mixed with primer 6 and primer 5 in a 9:1 molar ratio. After 25 cycles of amplification, 1 µl of the reaction was reamplified under the same conditions but using primer 16 as sense primer. A basic outline of this protocol is shown in figure 2.

Amplification of the 3' end of mRNA with one sequence specific primer and oligo dT : Three micrograms of total RNA or 0.5 µg of poly A+ RNA were reverse transcribed using the oligo dT primer 5. Before the addition of reverse transcriptase the sample was heated at 95°C for 10 min, 55°C for 10 min followed by 10 min at 4°C. The addition of this step improved the yield of

Figure 2. Five prime specific one-sided PCR method. Scheme of the basic protocol for the amplification of the 5' end of the MHV receptor using RNA as starting material. RES: restriction enzyme recognition site.

ONE-SIDED PCR

Cloning of the 5' End of a cDNA with 3' Sequence Information



product. The cDNA obtained was amplified in a standard PCR reaction using a 9:1 molar ratio of primers 6 and 5 and the sequence specific primer. The basic protocol is shown in figure 3.

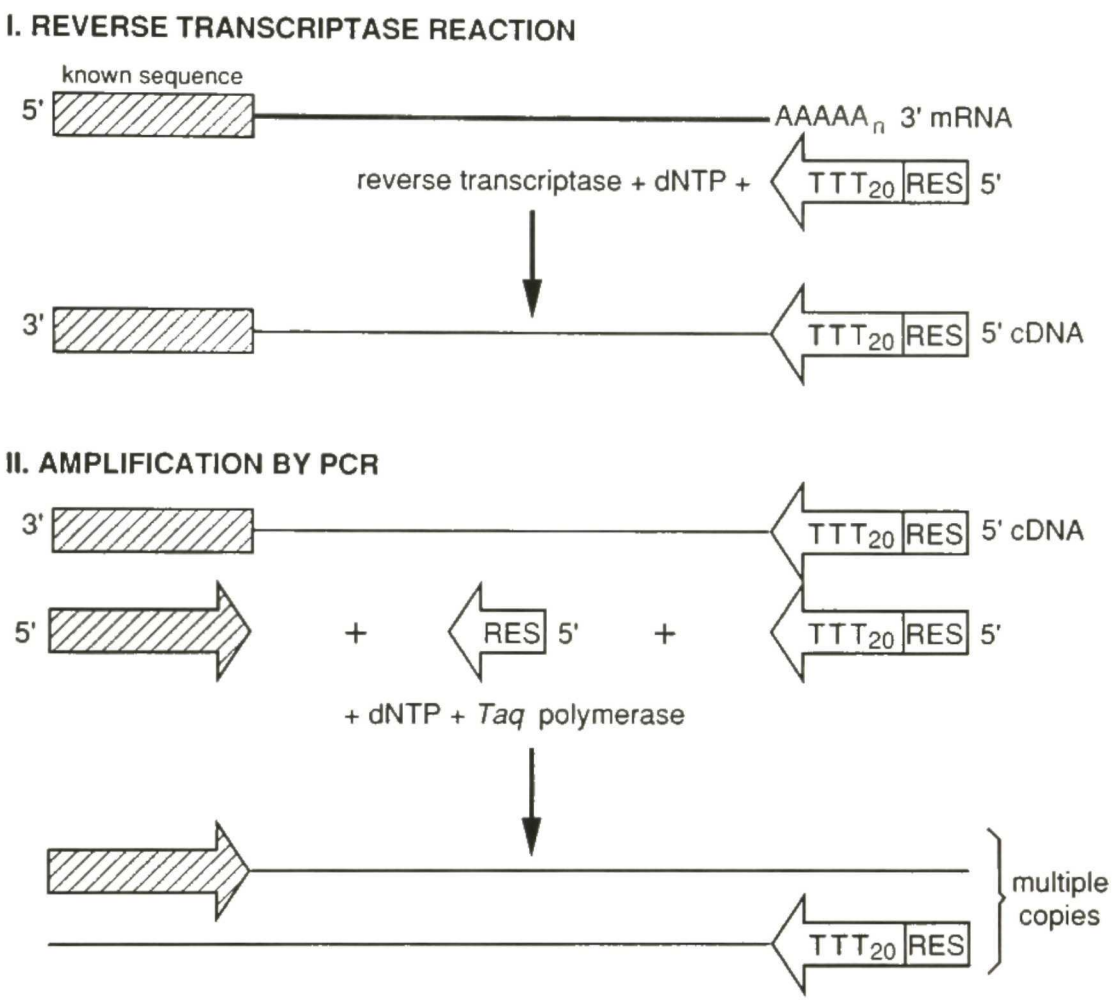
Thermocycling conditions : The Perkin Elmer/Cetus thermal cycler was used for all the amplification reactions. General cycling conditions were established in our laboratory. These are altered depending upon the size of the region to be amplified and the composition of the oligonucleotide primers. For amplification of regions of less than 500 bp the conditions of 94°C for 90 sec, 5° below the melting temperature of the primer of the lower melting temperature for 90 sec, 72°C for 90 sec for 25-30 cycles were followed. For degenerate oligonucleotide primers or primers containing restriction sites at the 5' end the annealing temperature was between 37° to 42°C for the first three cycles and was raised to the standard parameters for the rest of the amplification. For PCR products larger than 500 bp, the extension time at 72°C was 3 to 7 min to provide sufficient time for the completion of the DNA synthesis step in the reaction.

Alkaline agarose gels : To check the size of the first cDNA strand synthesized by reverse transcriptase an alkaline agarose gel was run according to the protocol described by Mc Donnell et al., (1977). One microgram of polyA+ RNA was reverse transcribed with primer 5 in a 50 µl reaction with 200 units of MMLV reverse transcriptase as recommended by the manufacturer. Two and a half µCi of α [^{32}P] dCTP were added to label the cDNA. After 1 hour at 37°C, the

Figure 3. Three prime specific one-sided PCR method. Scheme of the basic protocol for the amplification of the 3' end of a cDNA using RNA as starting material. RES: restriction enzyme recognition site.

ONE-SIDED PCR

Cloning of the 3' End of a cDNA with 5' Sequence Information



reaction was stopped by the addition of 2 µl of 0.5 M EDTA and diluted with dH_20 to 100 µl. The cDNA was precipitated overnight at (-20)°C with 0.1 volumes of 3 M sodium acetate and 2.5 volumes of ethanol. After centrifugation at 10,000xg the pellet was washed with 70% ethanol and dissolved in 20 µl of alkaline loading buffer (50 mM NaOH, 1 mM EDTA, 2.5 % Ficoll and 0.025 % bromocresol green). The sample was heated at 70°C for 15 min before loading 4 µl of it in a 0.8% agarose gel dissolved in 5 mM NaCl and 1 mM EDTA. The electrophoresis buffer composition was 30 mM NaOH and 1 mM EDTA. After the dye migrated approximately 8 cm, the marker lane was separated and neutralized for 1 hour in 1 M Tris.Cl pH 7.6, 1.5 M NaCl before ethidium bromide staining. A picture of the marker lane was taken with a fluorescent ruler to be used as a size reference. The rest of the gel was soaked for 30 min at room temperature in a solution of 7% trichloracetic acid and dried for several hours under many layers of Whatman 3MM paper between glass plates. The dried gel was covered with Saran Wrap and autoradiographed with an intensifying screen.

Analysis of MHV receptor transcript : BALB/c and SJL/J small intestine and colon RNA were electrophoresed on a 1.2% agarose gel after denaturation with glyoxal and dimethylsulfoxide (DMSO) by the method described by Thomas, (1980). In a sterile microfuge tube 10 µg of RNA in 7 µl of RNase free H_20 were

mixed with 6 µl of 10 M urea, 19 µl of DMSO and 6 µl of 6 M deionized gyoxal. The same reagents were added to 5 µl of the 1 kb ladder (Life Technologies) to be used as molecular weight marker. The RNA solutions were incubated for 1 hour at 50°C and rapidly cooled on ice. One microliter of gel loading dye (50% glycerol, 0.25% bromophenol blue) was added to each sample. The agarose was dissolved in 10mM sodium phosphate pH 7.0. The electrophoresis tanks were cleaned with abSolve (DuPont) and rinsed with a solution of 0.1% diethyl pyrocarbonate (DEPC). The gel was submerged and run in 10 mM sodium phosphate pH 7.0 at 3-4V/cm with constant recirculation of the sodium phosphate using a peristaltic pump to maintain the pH below 7.0. The RNA was transferred from the gel to a nytran membrane by capillary elution using solution HETS (Cinna/Biotecx) which allows visualization of the membrane-bound RNA under UV light. Following transfer with HETS, the side of the membrane containing the RNA was exposed to a source of ultraviolet irradiation (254 nm) for 2 min and baked for 1 hour at 80°C in a vacuum oven. The dried membrane was illuminated with UV light and the position of the ribosomal RNA's marked. The use of HETS for the transfer also permitted the visualization of the integrity of the RNA. The nytran membrane was submerged in a boiling solution of 20mM Tris.Cl pH 8.0, 0.1 % SDS. Following cooling the blot was prehybridized in 5X Denhardt's (for 500 ml of a 100X: 10 g Ficoll 400, 10 g polyvinylpyrrolidone and 10 g bovine serum albumin),100 µg/ml of carrier yeast

tRNA, 50 % deionized formamide, 5X SSPE (0.18 M NaCl, 0.01 M $NaPO_4$ pH 7.7, 1 mM EDTA) and 0.1 % SDS. Hybridization to the nick translated probes (10^6 cpm/ml of hybridization solution) took place overnight in the same solution and the filter was washed at a final stringency of 0.1% SSPE, 0.1% SDS at 60°C. The membrane was exposed to X-ray film with intensifying screens for the time indicated in the results section.

Sequencing of plasmid DNA : Double stranded sequencing of RNA-free minipreps or double banded cesium chloride purified plasmid was performed using the Sequanase kit (US Biochemicals). Briefly, 4 µg of plasmid in a volume of 18 µl were denatured with 2 µl of 2 M sodium hydroxide for 5 min. The DNA was then precipitated with the addition of 8 µl of 5M ammonium acetate pH 7.5 and 2.5 volumes of ethanol at -70°C for 15 min. After centrifugation the pellet was washed with 70% ethanol and redissolved in a final volume of 10 µl containing sequenase reaction buffer and 10 ng of the sequencing primer specific for that reaction. The mixture was heated at 65°C for 2 min and the primer and template were allowed to anneal by slow cooling at room temperature for 30 min. After the 30 min 1 µl of 0.1 M DTT, 2 µl of a 1:5 dilution of the labeling mix, 0.5 µl of [^{35}S] dATP (10 mCi/ml) and 3.25 units of sequenase were added and the reaction proceeded for 3 to 5 min at room temperature. Three and a half microliters of this reaction were added to four

different tubes, each tube had 2.5 µl of the specific termination mix. The reactions were allowed to take place for 3 min at 42°C and terminated by addition of 4 µl of stop solution. Before electrophoresis, the samples were heated at 80°C and rapidly cooled on ice. The 5% sequencing gel was poured and allowed to warm for at least two hours before sample loading. After electrophoresis the gel was transferred to a 3 MM Whatman paper, covered with Saran Wrap and dried for 60 min before exposing directly to film.

The primers used for sequencing were the forward primer provided in the sequencing kit, the reverse sequencing primer purchased from New England Biolabs and primers based on approximately the last 30 bases of the last sequence information obtained. In this way, the two DNA strands were sequenced and compared for verification.

Oligonucleotide labeling : The standard procedure for labeling oligonucleotides to use as probes was the following. One microgram of the oligonucleotide was incubated for 30 min at 37°C in the presence of 200 to 250 µCi of γ [^{32}P] ATP, 10 units of T4 polynucleotide kinase and polynucleotide kinase buffer (10X: 0.5 M Tris.Cl pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM EDTA). The reaction was stopped by the addition of 2 µl of 0.5 M EDTA pH 8.0. The labeled oligonucleotide was employed at a concentration of 10 ng/ml of hybridization solution (5X SSPE, 5X Denhardt's and 0.1 % SDS) and stored up to a week at -20°C.

Library screening : The lambda gt11 cDNA libraries and the genomic library were screened according to the protocol first described by Benton and Davis, (1977). Six hundred thousand plaques were screened from each library. Three rounds of screening of duplicate nitrocellulose filters were necessary to isolate single positive plaques. The probe used for hybridization was either a plasmid insert purified from an agarose gel using the geneclean kit (Bio 101) and labeled by nick translation (Life Technologies) or a mixture of end labeled oligonucleotides . After nick translation labeling, the probe was purified with a Nick column (Pharmacia), counted and diluted to 10^6 cpm/ml of hybridization solution. The hybridization was performed overnight using 5X Denhardt's, 100 µg/ml of denatured, fragmented salmon sperm DNA, 50 % deionized formamide, 5X SSPE and 0.1 % SDS. The filters were washed extensively in 1 X SSPE, 0.1 % SDS at 65°C. When oligonucleotides were employed as probes the unincorporated label was removed by passing the reaction through a NACS column (Life Technologies) and the probe was used at a concentration of 10 ng/ml of hybridization solution. The formamide and carrier DNA were eliminated from the hybridization solution when using oligonucleotide probes and the washes were performed at 5°C below the calculated melting temperature of the oligonucleotide in 5X SSPE, 0.1 % SDS. The simple equation Tm= 4(G+C) + 2(A+T) was used for oligonucleotides less than 20 bases long. The autoradiogram of the positive nitrocellulose replica were aligned with the original plaque plate and plaques corresponding to positive signals picked. The

phage were eluted by incubation of the agarose plugs in phage storage buffer (50 mM Tris.Cl pH 7.5, 70 mM NaCl, 10 mM $MgSO_4$ and 0.1% gelatin) containing 0.1 volumes of chloroform, stored at 4° C and then titered for rescreening.

The cosmid library was screened with the nick translated probe following the instructions provided by the manufacturer (Stratagene).

Analysis of genomic DNA : Ten micrograms of BALB/c or SJL/J liver genomic DNA was digested for 5 hours with either 4 units/ µg of EcoR I or Sst I. The digested DNA was electrophoresed on a 0.8% agarose gel and transferred overnight by capillary elution using 20 X SSPE to a nytran membrane as described by Southern, (1975). To facilitate the transfer of high molecular DNA species, the gel was placed under UV light for 5 min. The position of the gels slots were marked on the filter and the gel restained with ethidium bromide (0.5 µg/ml in 0.1 M ammonium acetate) to verify the efficiency of the transfer. Following transfer the nytran membrane was soaked in 6X SSPE to remove any pieces of agarose sticking to the filter. The DNA was UV cross-linked and the membrane baked for 1 hour at 80°C in a vacuum oven. The blot was hybridized to the radiolabeled probe under the same conditions described for library screening. The nick translated probe was washed at a final stringency of 0.1% SSPE, 0.1% SDS at 55°C.

In vitro transcription and translation : Production of capped mRNA was

performed using pMHVRE. This plasmid was linearized with the restriction enzyme Not I. The purified linear plasmid (1 µg) was incubated with transcription buffer (40 mM Tris HCl pH 8.0, 8 mM $MgCl_2$, 50 mM NaCl, 2 mM spermidine, 30 mM DTT), 400 mM of each rNTP, 40 mM of 5'7MeGpppG and 10 units of T7 RNA polymerase in a 25 µl reaction for 60 min at 37°C. Trace amount of α [^{32} P] UTP was added to the transcription reaction and the synthesized mRNA was sized by gel electrophoresis. The in vitro translations were performed in rabbit reticulocyte lysates. The manufacturer's instructions were followed for the generation of [^{35} S]- methionine labeled protein. Briefly, the template mRNA was heated at 70°C for 10 min and immediately cooled on ice to destroy regions of secondary structure. Ten micrograms of the in vitro transcribed RNA were incubated for 1 hour at 30°C with 40 units of RNasin ribonuclease inhibitor (Promega), 35 µl of nuclease treated lysate, 1 µl of 1mM amino acid mixture minus methionine and 4 µl of [^{35}S]- methionine (10 mCi/ml). Brome mosaic virus RNA provided in the kit was translated in a different reaction as a positive control. A 2 µl aliquot was removed from the translation reaction. To analyze the incorporation of the labeled amino acid the removed aliquot was TCA precipitated and the counts were determined on a filter paper as recommended by the manufacturer. Proteins were separated on SDS-polyacrylamide gels by the method of Laemmli, (1970).

Immunoprecipitation of the MHV receptor : For analysis of direct translation products 10 µl reticulocyte lysate was denatured with sample treatment mix (62.5 mM Tris HCl pH 6.8, 2.3% [wt/vol] SDS, 5% [vol/vol] β mercaptoethanol, 10% [vol/vol] glycerol). Samples for analysis were electrophoresed on 10 % PAGE gels. Following electrophoresis, the gels were fixed with methanol:glacial acetic acid and stained with Coomasie brilliant blue R250. The gels were destained in methanol:acetic acid and dried in vacuo before exposing to X-ray film. For immunoprecipitation of the translated protein, nonspecific precipitates were pre-cleared by incubation of the in vitro translated protein with preimmune serum for 2 hours at room temperature, followed by 100 µl of 10 % (wt/vol) protein A-Sepharose beads (Pharmacia) for 1 hour at room temperature with end-over-end agitation. Precipitates were washed three times in RIPA buffer (0.1 M NaCl, 1 mM EDTA, 0.01 M Tris.Cl pH 7.4, 0.1 % NP40) and saved to run on the gel. The supernatant was transfered to a new tube and the radiolabebelled proteins were specifically immunoprecipitated with polyclonal rabbit antibody directed against affinity purified receptor. After 2 hours, protein A-Sepharose beads were added and the immunoprecipitates were washed as indicated above. The supernatant and the specific precipitate were mixed with sample buffer and analyzed by SDS-11.5 % PAGE gel electrophoresis. The gel was stained, destained and autoradiographed as described above.

ESTABLISHMENT OF A PCR-BASED CLONING SYSTEM

Introduction

The most commonly used procedures in gene isolation require the construction of a cDNA library from the RNA of tissue or cells. An antibody or a nucleic acid probe can be used to screen this library for the gene of interest (Suggs et al., 1981; Young and Davis, 1983). These two approaches have been successful in cloning a great number of genes. In some cases, previous knowledge of a short stretch of the nucleotide sequence of the gene of interest is enough to design an oligonucleotide to be used as a radiolabeled probe. The more nucleotide sequence information, the easier is the process of screening to obtain the desired gene. With the use of expression libraries, expression of fusion proteins can be induced and the peptide identified with polyclonal or monoclonal antibodies. Identification of the phage encoding for the peptide ultimately leads to the sequence of the cDNA.

Results and Discussion

First attempts for cloning the mouse hepatitis virus receptor: Both classical approaches have been attempted for the cloning of the mouse hepatitis virus (MHV) receptor without success. The MHV receptor has been purified to homogeneity from liver membranes and microsequenced (Williams et al., 1990). The identification of 15 amino acids of the receptor protein made possible the synthesis of an oligonucleotide probe for screening. This

oligonucleotide is 35 nucleotides long, spans the sequence of the first twelve amino acids of the mature receptor protein and is 8-fold degenerate (primer 1 in Table 3). The third position of each amino acid was selected using a rodent codon usage table for glycoproteins. Two positive clones were obtained when screening an NIH 3T3 cDNA library with this oligonucleotide probe (R. Williams, personal communication). A polyclonal antibody was raised against a synthetic peptide comprising the first 15 amino acids of the receptor (Williams et al., 1990). This antibody was employed in the screening of an expression library. After three rounds of screening of a BALB/c mouse liver lambda gt11 expression library (Promega) twelve positive clones were isolated (R. Williams, personal communication). The clones obtained from both libraries were divided into groups according to their pattern of cross-hybridization. Nucleotide sequencing of the largest insert of each group was performed. The sequence coding for the first 15 amino acids of the receptor was not present in any of the phage inserts analyzed.

Screening of a JLSV9 cDNA library (constructed by Dr Dieffenbach), a B6/CBA mouse liver lambda Zap cDNA library (Stratagene) and a Mbo I partial BALB/c liver embryonic genomic library (constructed by Dr Seidman) with the oligonucleotide probe did not identify any possible receptor clones.

Development of an internal control for RNAPCR : The use of RNA-polymerase chain reaction (RNAPCR) as a tool for cloning cDNA molecules was introduced in the past two years (Ohara et al., 1989; Frohman et al., 1988; Loh et al., 1989). RNAPCR is a technique that permits detection and analysis of mRNA

expression (Rappolee et al., 1988; Jacobsen et al., 1989; Brenner et al., 1989). This procedure requires the synthesis of cDNA by reverse transcriptase from the mRNA template. Once the desired cDNA molecule is present, there is little difference between RNAPCR and the regular DNA-based polymerase chain reaction.

The development of RNAPCR to clone genes that are expressed in very low levels gave the opportunity of a new method for cloning the MHV receptor. When we decided to try this technique for cloning purposes, few reports could be found in the literature. Due to the lack of a standardized approach and the novelty of the technique, the use of an internal control to monitor each step was of importance for the success of the project. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the logical choice for this role since it has served as an excellent internal control for Northern blots (Dieffenbach et al., 1989). GAPDH is a house keeping gene and its level of expression is independent of the cell cycle. A set of primers to be used for PCR amplification of the GAPDH cDNA were developed in our laboratory. The sequences of the sense, antisense primer, and the oligonucleotide used as a probe and their positions in the human cDNA is shown in figure 4. The design of a probe that hybridizes to a sequence between the amplification primers discriminates the possible non-specific PCR products that can be generated in a reaction. The non-specific products obtained are in general the result of the primer-dimer phenomenon, the low annealing temperature employed in the PCR reaction or the use of degenerate oligonucleotide primers. Primer-dimer refers to the short products

Figure 4. Nucleotide sequence of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) human cDNA. The position of the primers and the distance to the poly A tail is shown. The sequence of the sense and antisense primer is in bold and the sequence of the probe is underlined. Note that the complement of the highlighted antisense primer was actually synthesized and used.

CATGGGGAAGGTGAAGGTCGGAGTCAACGGATTTGGTCGTATTGGGCGCC 50

TGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGTGGATATTGTTGCCATC 100

AATGACCCCTTCATTGACCTCAACTACATGGTTTACATGTTCCAATATGA 150

TTCCACCCATGGCAAATTCCATGGCACCGTCAAGGCTGAGAACGGGAAGC 200

TTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAGCGAGATCCCTCC 250

AAAATCAAGTGGGGCGATGCTGGCGCTGAGTACGTCGTGGAGTCCACTGG 300

CGTCTTCACCA**CCATGGAGAAGGCTGGGG**CTCATTTGCAGGGGGGAGCCA 350

AAAGGGTCATCATCTCTGCCCCCTCTGCTGATGCCCCCATGTTCGTCATG 400

GGTGTGAACCATGAGAAGTATGACAACAGCCTCAAGATCATCAGCAATGC 450

CTCCTGCACCACCAACTGCTTAGCACCCCTGGCCAA**GGTCATCCATGACA** 500

ACTTTGGTATCGTGGAAGGACTCATGACCACAGTCCATGCATCACTGCCA 550

CCCAGAAGACTGTCGATGGCCCCTCCGGGAAACTGTGGCGTGATGGCCGC 600

GGGGCTCTCCAGAACATCATCCCTGCCTCTACTGGCGCTGCCAAGGCTGT 650

GGGCAAGGTCATCCCTGAGCTGAACGGGAAGCTCACTGGCATGGCCTTCC 700

GTGTCCCCACTGCCAACGTGTCAGTGGTGGACCTGACCTGCCGTCTAGAA 750

AAACCTGCCAAATATGATGACATCAAGAAGGTGGTGAAGCAGGCGTCGGA 800

GGGCCCCCTCAAGGGCATCCTGGGCTACACTGAGCACCAGGTGGTCTCCT 850

CTGACTTCAACAGCGACACCCACTCCTCCACCTTTGACGCTGGGGCTGGC 900

ATTGCCCTCAACGACCACTTTGTCAAGCTCATTTCCTGGTATGACAACGA 950

ATTTGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCACATGGCCTCCA 1000

AGGAGTAAGACCCCTGGACCACCAGCCCCAGCAAGAGCACAAGAGGAAGA 1050

GAGAGACCCTCACTGCTGGGGAGTCCCTGCCACACTCAGTCCCCCACCAC 1100

ACTGAATCTCCCCTCCTCACAGTTGCCATGTAGACCCCTTGAAGAGGGGA 1150

GGGGCCTAGGGAGCCGCACCTTGTCATGTACCATCAATAAAGTACCGGAA 1200

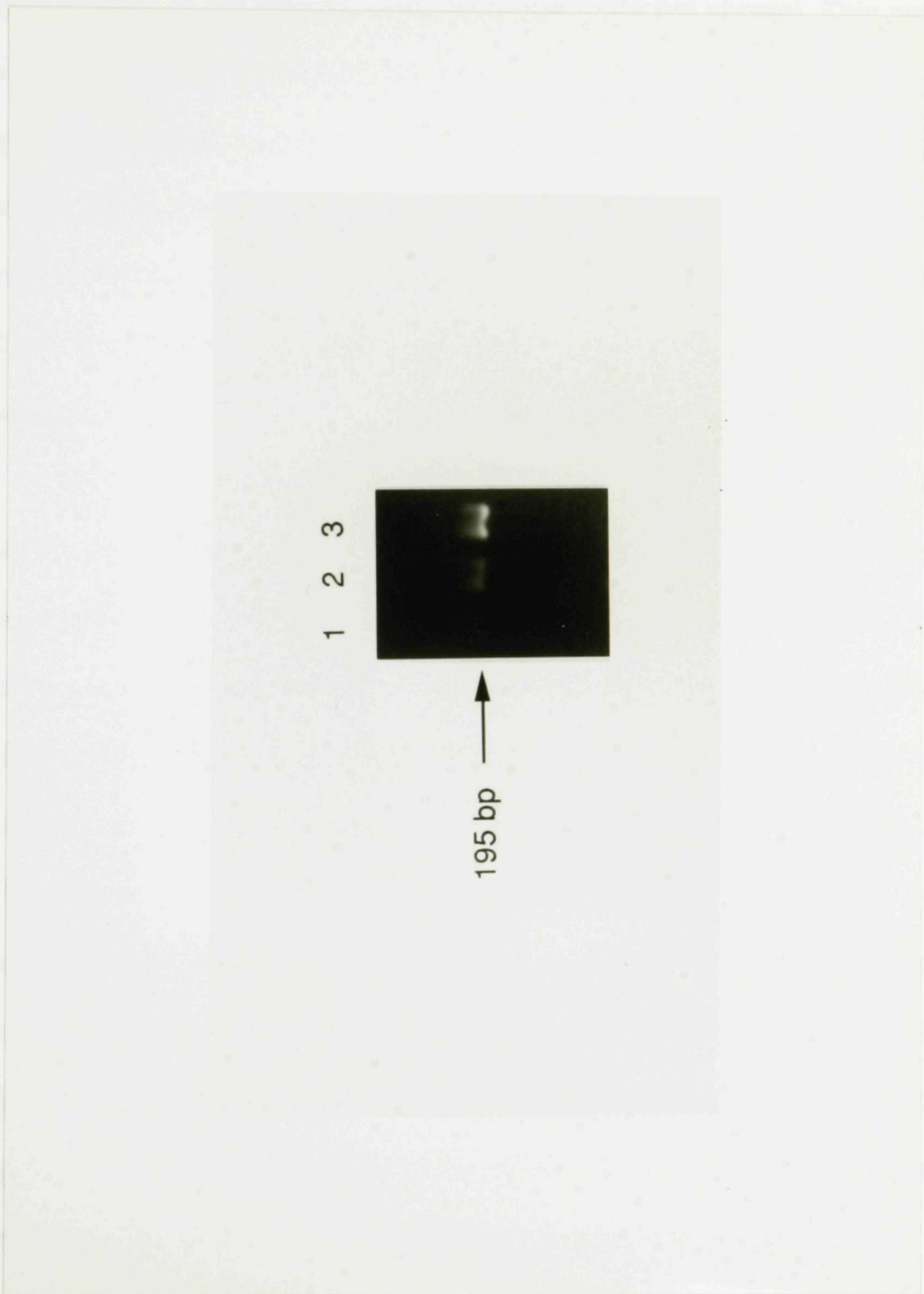
TTC(AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA)

that are generated independently of added template DNA and are the result of the extension of each primer (Watson, 1989). The primer-dimer may occur when the 3' ends of each primer contain two or more complementary bases which allow for hybridization and the formation of a primed template complex. Low annealing temperatures permit hybridization of the primers to non-specific, partially complementary sequences. The use of degenerate oligonucleotides gives the possibility of amplification of several regions of the cDNA (Strub and Walter, 1989). This can occur if there is enough sequence complementarity at the 3' end of the oligonucleotide to permit the annealing of the primers of the pool to different parts of the template.

The specificity of the product obtained can be verified with the oligonucleotide probe. Southern blot analysis of the gel after electrophoresis of a small percentage of the amplified material and hybridization to the specific probe is essential to avoid misleading results (Saiki et al., 1986; Kerem et al., 1990). Other researchers approached this problem with different strategies. Rappolee designed primers around diagnostic restriction sites, so digestion of the PCR product with the appropriate enzyme resulted in the production of two bands of a precise size (Rappolee et al., 1988; 1989).

The selection of primers from the nucleic acid sequence of interest is critical to the success or failure of the PCR. To test the primers chosen in our laboratory based on the published GAPDH sequence, linearized plasmid containing the human GAPDH cDNA sequence was amplified. Different concentrations of template were employed as shown in figure 5. The amplified

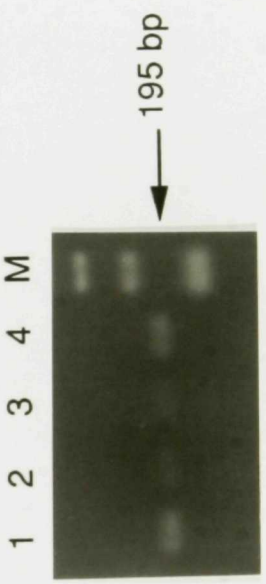
Figure 5. Plasmid DNA concentration dependence of amplification of glyceraldehyde-3- phosphate dehydrogenase (GAPDH). A Pst I linearized plasmid containing the GAPDH cDNA clone was boiled and diluted to a concentration of 0.01 ng (lane 1), 0.1 ng (lane 2) and 1 ng (lane 3). This plasmid DNA was amplified with primers 2 and 3 listed in Table 3 for 20 cycles as described in the Methods section. A photograph of the ethidium bromide stained gel where 10 µl of each reaction was electrophoresed is shown.


1 2 3
195 bp

material was Southern blotted and shown to be specific for GAPDH by hybridization to the internal GAPDH probe (data not shown). This set of primers was examined in an RNAPCR reaction with RNA of different mammalian species including human, rat, mouse, and chicken (Figure 6). The amplification products can be detected with the same probe shown in figure 4 for all three mammalian species but not chicken (data not shown). Detection of the chicken GAPDH product uses a different probe due to the difference in the nucleotide sequence between the primers employed.

Classical PCR amplification requires enough sequence information to design two primers, one sense and one antisense at a distance compatible with amplification by Taq polymerase. The optimal distances are 140 to 1500 bp, although amplification products up to 10 kb have been reported (Jeffrey et al., 1988). Because of the absence of enough information to design an antisense primer, a different scheme for the receptor amplification had to be considered. This approach is illustrated in figure 3 and makes use of the poly A tail present in most eukaryotic mRNAs to prime the reverse transcriptase reaction. All the mRNA molecules are copied to cDNA, and the specificity in the amplification reaction is determined by the sense primer employed. We decided to try this approach for the amplification of the MHV receptor. To determine the optimal conditions for amplification, the GAPDH system was employed. The distance between the sense primer and the poly A tail of the mRNA that codes for GAPDH is shown in figure 4 and is approximately 1kb. We anticipated that the amplification of the MHV receptor was going to be more inefficient than that of

Figure 6. Demonstration of amplification of GAPDH from RNA of different animal species. Reactions for RNAPCR using 1 μg of total cellular RNA from human fibroblast (lane 1), mouse L929 (lane 2), rat fibroblast (lane 3), and chicken epithelium (lane 4) were performed as described in the Methods section using primers 2 and 3 listed in Table 3. The molecular size marker is the 123 bp ladder purchased from Life Technologies (lane M). The photograph shows the ethidium bromide stained gel after electrophoresis of 10% of the total PCR reaction.


195 bp
M
4
3
2
1

GAPDH for several reasons. First, the sense primers for MHV are degenerate oligonucleotides, and the perfect match might be absent from the mix. Second, the number of mRNA copies per cell of the GAPDH message was expected to be much more greater than the mRNA coding for the receptor, and finally, the distance between the sense primer and the poly A tail of the receptor was expected to be larger than 1 kb based on the size of the receptor protein. Increased distance between primers may result in a drop in efficiency of the PCR process and less product is obtained per cycle. To facilitate the subcloning process, restriction enzyme recognition sites were added to the oligo dT antisense primer (primer 5 in Table 3). The restriction enzyme recognition sites were employed as a second antisense primer in the PCR reaction (primer 6 in Table 3). The length of oligonucleotide 5 decreases its efficiency to act as a primer in the amplification. The sequence of the shorter primer will be incorporated in all the cDNA molecules after priming the reverse transcriptase reaction with oligonucleotide 5 and is expected to be more efficient. The Not I recognition sequence was chosen due to the low probability of having an internal site within the receptor cDNA. When total RNA prepared from JLSV9 cells was used for the amplification of the GAPDH cDNA no product was detected after electrophoresis of 10 to 20% of the reaction. When the electrophoresed product was analyzed by Southern blot and probed, a specific GAPDH band could be detected. This band corresponded to the predicted distance between the sense primer and the 3' end of the transcript. A special scheme for annealing of the primer for the cDNA reaction had to be developed

for the reverse transcriptase reaction. It consisted of the successive incubation of the sample for 10 min at 94°C, 5 min at 55°C and rapid cooling on ice before addition of the reverse transcriptase.

Because of the need to visualize the product on an ethidium bromide stained gel for further subcloning of the receptor, poly A+ RNA isolated from JLSV9 was employed in the RNAPCR to obtain better yields. As shown in figure 7, the amplification product was visualized in the stained agarose gel and the product was shown to be specific after hybridization to the GAPDH probe.

Since degenerate oligonucleotide primers had to be employed in the amplification of the MHV receptor cDNA, the minimun amount of sense primer necessary for successful amplification had to be determined. Figure 8 shows that less than 150 ng of GAPDH sense primer result in absence of visible product. The same approach was attempted with the 8 fold degenerate oligonucleotide (primer 1 in Table 3) based on the receptor sequence that was designed for library screening. RNA prepared from confluent (quiescent), subconfluent (log phase) and just confluent cells was used for the amplifications because no information of receptor expression at different growth status is available. Amplification with this oligonucleotide as sense primer did not yield a PCR product after 30 cycles using a variety of annealing temperatures. One possible explanation is that the length of the primer exceeds the recommended one for optimal amplification. Another bad feature of this oligonucleotide has been revealed to us after the analysis of the actual nucleotide sequence that codes for the MHV receptor. The oligonucleotide has three mismatches with the

Figure 7. Production of a GAPDH PCR product visible by agarose gel electrophoresis from poly A+ RNA. Poly A+ RNA (0.5 μg) was reverse transcribed with primer 5. The cDNA was amplified using primer 2 and primers 5 and 6 for 25 cycles. The upper band (approximately 950 bp) that is detected by ethidium bromide staining in A and blot hybridization in B is the accurately produced product. The 500 bp band arises by an additional antisense priming event where the restriction enzyme oligonucleotide acts as antisense primer at position 876 in the GAPDH cDNA. The sequence of the oligonucleotides employed is listed in Table 3.

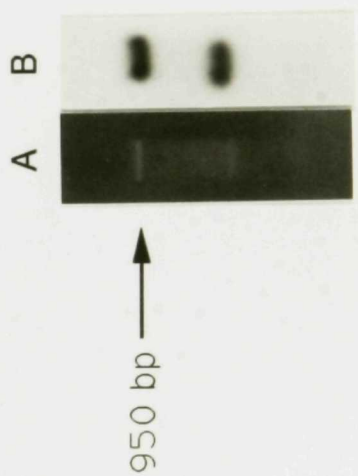
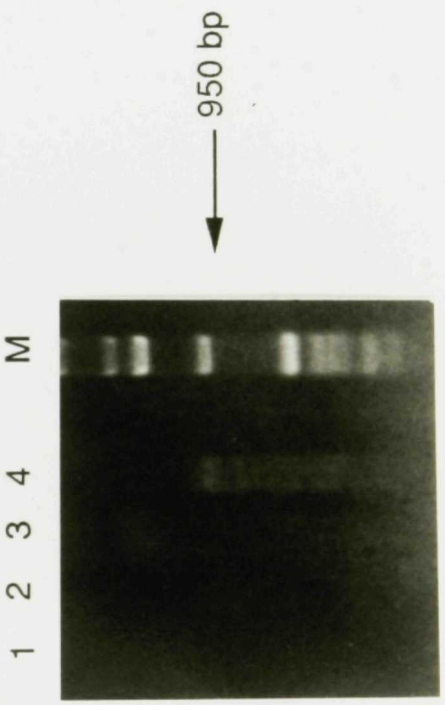

B
A
950 bp

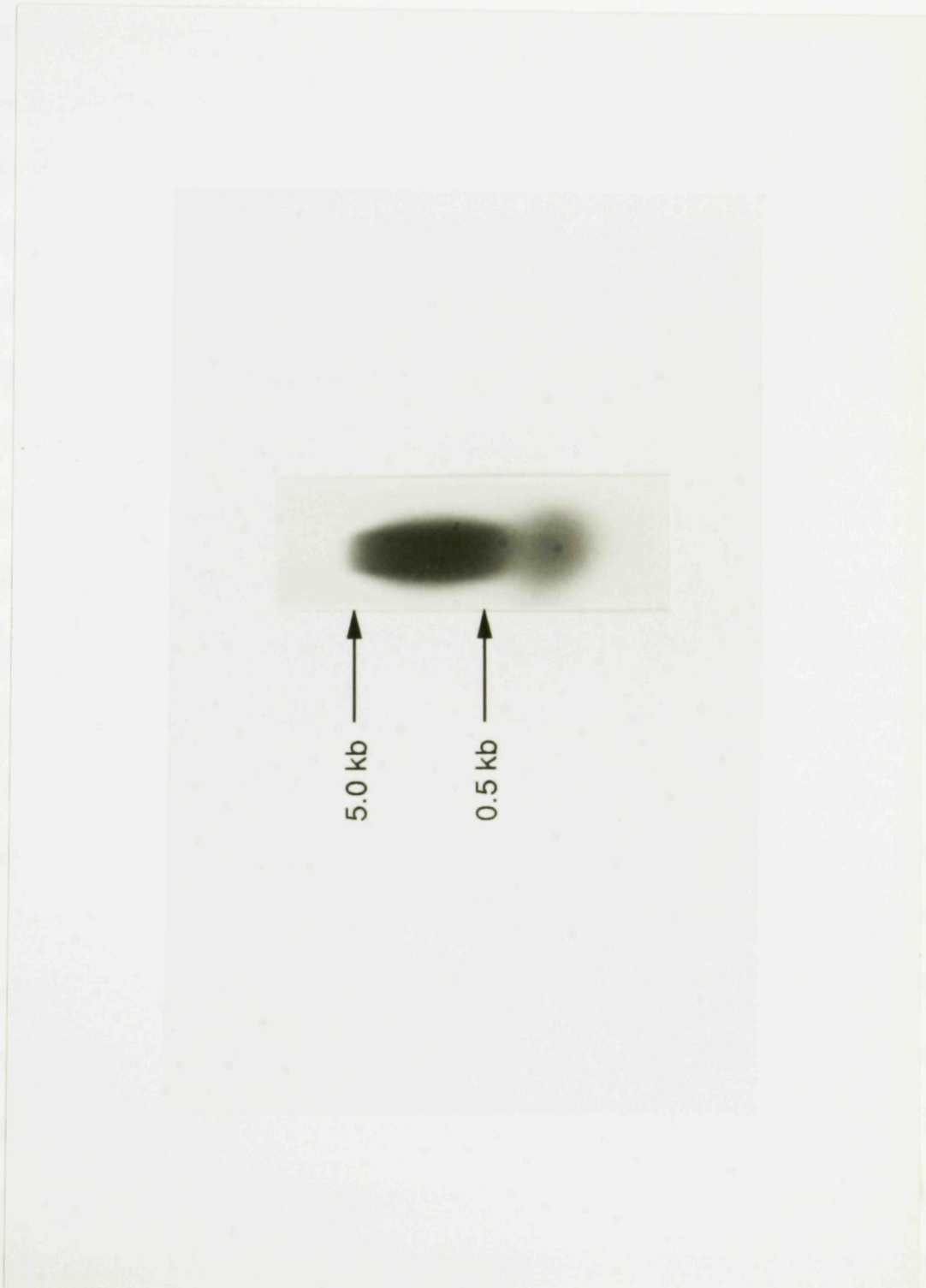
Figure 8. Titration of sense primer requirements for successful GAPDH amplification. As a test for the quantity of sense primer required for amplification of oligo dT primed GAPDH cDNA, amplifications were performed with 1 μg of the combined mixture of primers 5 and 6 as described in the Methods section and different amounts of primer 2 : 10 ng (lane 1), 25 ng (lane 2), 50 ng (lane 3), and 150 ng (lane 4). The primer sequences are listed in Table 3. Lane M corresponds to the 1 kb ladder (Life Technologies) employed as molecular size marker. The PCR was performed for 25 cycles. A photograph of a 1% agarose gel following electrophoresis of 20% of the product is shown.


950 bp
M
1 2 3 4

receptor sequence, they correspond to the third position of the codons and are bases number 6, 24 and 33 in the primer. Although the first two mismatches are probably not critical, the mismatch to the template in position 33 of this 35 nucleotide long primer is of importance. The absence of base pairing between primer and template corresponding to the third base of the primer starting from its 3' end is likely to preclude the possibility of extension by the enzyme. The minimal homology requirement between template and primer for successful amplification has been carefully analyzed by Sommer and Tautz, (1989). They concluded that the length of the primer should be preferably 20-24 nucleotides and that the three 3' nucleotides should match completely. At the moment of designing the oligonucleotide, the base selected for that position was the one for the codon most highly represented according to the rodent codon usage table created by Dr. F. Jenkins. The codon present in our clone for that amino acid is only used 15% of the time and is absent in the oligonucleotide employed.

Another possibility for the failure of the amplification was the inability of the reverse transcriptase to make cDNA molecules of great enough length without falling off the template. The inability of the enzyme to extend the cDNA molecule from the antisense primer to the sequence complementary to the receptor-specific primer employed, will determine the absence of amplified product. This hypothesis was tested by analysis of the synthesized cDNA in the presence of labeled dCTP and electrophoresis of the sample (Figure 9). The cDNAs synthesized reached 5 kilobases. This size was considered more than

Figure 9. Determination of cDNA product sizes by alkaline agarose gel electrophoresis. An oligo dT (500 ng of primer 5 in Table 3) primed cDNA synthesis reaction was supplemented with 2.5 μCi of α [^{32}P]dCTP. Following synthesis at 37°C for 1 hour the reaction was diluted to 100 μl and ethanol precipitated. After resuspension of the nucleic acid pellet, the sample was processed as described in the Methods section. An autoradiogram of the dried agarose gel is shown.


5.0 kb
0.5 kb

the expected for the receptor transcript.

Affinity purification of the MHV receptor using the monoclonal antibody CC1 was scaled up by Dr Jiang in Dr K. V. Holmes laboratory. The protein was sent to be microsequenced in a different laboratory and the first 25 amino acids were determined. Comparison with the receptor sequence previously obtained showed a disagreement for the amino acids corresponding to positions 8 and 9. The last 8 amino acids in the new sequence could not be used in the design of primers due to the multiple codon possibilities presented in that portion of the peptide. Three new primers were designed based on the first 17 amino acids for PCR amplification of the MHV receptor. These were shorter and more degenerate than the one described above. When designing these three new primers certain rules were applied to minimize their degree of degeneracy. A rodent codon usage table was employed, the position for the last amino acid in the primer was only represented by the first two nucleotides of the codon to guarantee perfect match between template and the 3' end of the primer, and the CpG sequence was avoided (Bird, 1986). The nucleotides coding for amino acids 8 and 9 were not included in the new set of oligonucleotides. Two of the primers (numbers 7 and 8 in Table 3) are based on amino acids 1 to 7. These primers are 64-fold degenerate and they differ in the third position from the 3' end. The use of these primers in separate reactions should guarantee perfect match of at least one of them between the end of the oligonucleotide and the template. The other primer (number 9 in Table 3) spans the sequence between amino acid 10 to 17 and is 32-fold degenerate. The three degenerate

oligonucleotides were employed as sense primers in three separate RNAPCR reactions. They were used in conjunction with the oligo dT based oligonucleotide in the same scheme described for the amplification of GAPDH. No product was obtained with RNA from the JLSV9 cell line. The amount of primer, annealing temperature, and cycle number were changed several times in an attempt to obtain the product. The reaction conditions and integrity of the RNA were verified by the simultaneous successful amplification of GAPDH. These was done in two different ways: the two cDNAs were co-amplified in the same tube after the cDNA reaction in the presence of the specific sense primers or they were divided in to separate tubes for further amplification. For this purpose a 30 µl reverse transcriptase reaction was performed in the presence of the antisense primer, after heat-inactivation of the reverse transcriptase at 95°C, the reaction was divided in two. Five microliters of the reverse transcriptase reaction was employed for the amplification of the GAPDH cDNA upon addition of the sense primer. The rest of the cDNA reaction was used for the amplification of the receptor with the degenerate primer.

We believe that the length of the expected product (approximately 1.45 kb) and the wrong choice for source of RNA contributed to the failure of this approach. Expression of the MHV receptor in JLSV9 cells was detected by immunofluorescence using a monoclonal antibody directed against the MHV receptor (MAbCC1) as well as demonstrated by its susceptibility to infection by MHV-A59 (C. Stephensen, personal communication). The level of expression of the receptor protein varies considerably in the different tissues of susceptible

animals (Williams et al., 1990). I found that the same variability is observed at the mRNA level when the receptor clone became available for these studies. Expression of the transcript of the MHV receptor in liver is very low compared to colon and small intestine. When amplification of the receptor RNA from liver was attempted using the degenerate oligonucleotides and a non degenerate internal receptor-specific antisense primer in an RNAPCR reaction, the band obtained was not visible in an ethidium bromide stained gel. Liver poly A+ RNA had to be employed for visualization of the product upon electrophoresis and ethidium bromide staining. The lack of an antisense specific oligonucleotide, and the need to use an oligo dT primer in the reactions attempted from JLSV9 RNA could explain the absence of detectable product if the level of expression of the receptor transcript in this cell line is comparable to the level of expression in liver of susceptible animals.

CLONING OF THE MHV RECEPTOR

Introduction

A search of the National biomedical research foundation (NBRF) data base for sequences related to the first 25 amino acids of the affinity purified receptor from Swiss Webster mouse liver membranes revealed homology between the MHV receptor and members of the human carcinoembryonic family of glycoproteins (Williams et al., submitted for publication). Various human members of the carcinoembryonic antigen (CEA) family have been cloned and characterized (Neumaier et al., 1988; Zimmerman et al., 1987; Hinoda et al., 1988; Khan and Hammarstrom, 1989; Khan et al., 1989). CEA, the best studied member of the family, is a highly glycosylated protein of molecular weight 180,000 and is expressed at greatly increased levels in nearly all human colon carcinomas (Shuster et al., 1980). It was first described by Gold and Freedman, (1965) as an antigen expressed in tumors of the human gastrointestinal tract and in the fetal digestive system. Immunoassay for CEA is a diagnostic tool in primary clinical diagnosis of colon cancer as well as in the long term monitoring of patients following colorectal tumor resection.

The original concept that CEA is only associated to tumorigenicity and embryonic life was abandoned when small amounts of CEA were found in normal adult colon (Egan et al., 1977; Fritsche and Mach, 1977). As other CEA family members are purified and cloned, their patterns of expression are defined. For example, another family member, the non-specific cross reactive

antigen (NCA), is produced in some colon, breast and lung carcinomas (Zimmermann et al., 1988; Cournoyer et al., 1988) and in normal lung and spleen (von Kleist et al., 1972; Mach and Pusztaszeri, 1972).

The exact number of genes in the CEA family is unknown. The number of human CEA-related genes appears to be in the order of twelve as indicated by Southern blot analysis with CEA probes (Thompson et al., 1987; Zimmermann, et al., 1988). Thirteen different CEA-related proteins have been identified biochemically or by molecular biology techniques (Buchegger et al., 1984; Kuroki et al., 1981; Matsuoka et al., 1982; Neumaier et al., 1985; von Kleist et al., 1972; Neumaier et al., 1988; Svenberg et al., 1979; Audette et al., 1987; Khan et al., 1989). The high degree of homology between the CEA related genes at the amino acid and nucleotide levels implies that they evolved rather recently by duplication of a primordial gene. It has been proposed that the various closely related members of the CEA gene family must have arisen by gene duplication and exon shuffling (Gilbert, 1978). Some members of the CEA gene family were shown to be the products of alternative RNA splicing; this phenomenon was described in the tumor cell line HT-29 and human fetal liver (Barnett et al., 1989) and for the three different forms of biliary glycoprotein I (BGPI) observed (Hinoda et al., 1988). This was demonstrated by hybridization of Northern blots with probes from defined regions within the BGP-I related transcripts. One probe hybridized to all three mRNA species while a second probe detected only the largest and smallest forms.

Sequence comparison of the cloned human CEA related genes

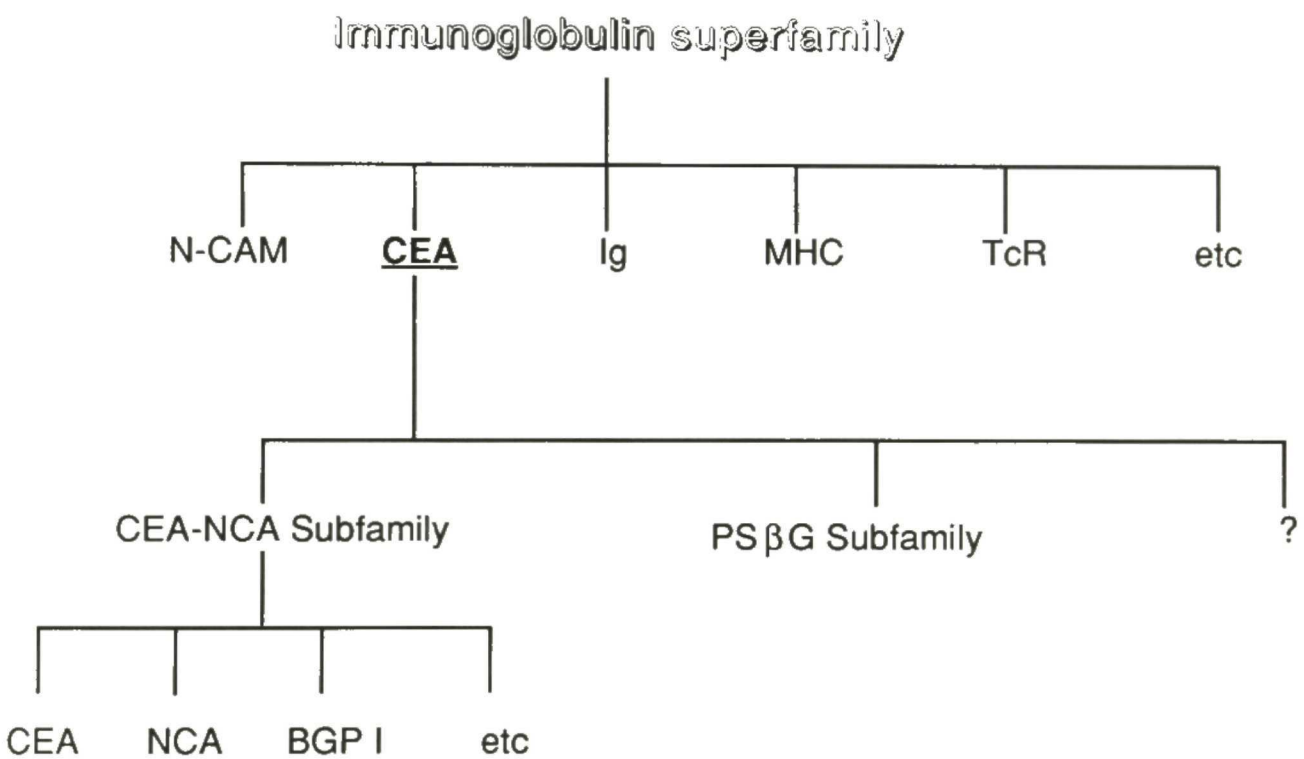
identified three different subgroups. One contains the genes encoding the classical CEA-related antigens. A second subgroup contains the genes which encode the pregnancy-specific glycoproteins. An additional gene whose product is unknown represents a third subgroup member (Thompson et al., 1989b) (Figure 10).

Several genes of the human and rat CEA family have been isolated and sequenced at their N-terminal domain exons. Interspecies comparison between the different rat and human CEA related genes shows a degree of nucleotide sequence similarity for the N-terminal domain exons of 52-66% (Kodelja et al., 1989). The degree of similarity within the human CEA-related genes is between 67% and 95%. The loss of similarity between species reveals a strong divergence between the human and rat families, indicating a parallel but independent evolution from one or more primitive genes, leading to different CEA gene families in the primate and rodent orders (Rudert et al., 1989). Analysis of the N-terminal domain exons of murine CEA-related genes has not been possible due to the lack of genomic sequence information.

When the secondary structure of the N-terminal domains of the human and rat CEA related proteins was analyzed by computer program, a close similarity to the variable domains of immunoglobulins could be determined (Thompson et al., 1989a). A specific number of β strands can be deduced which are characteristic for the immunoglobulin-like fold (Thompson et al., 1989a).

CEA has been suggested to function as an intercellular adhesion molecule and to play a role in the development and maintenance of the tissue

Figure 10. Family tree of the immunoglogulin superfamily as described by Thompson, et al., (1989). N-CAM, neural cell adhesion; Ig, immunoglobulins; MHC, major histocompatibility antigens; TcR, T cell receptors; NCA, nonspecific cross-reacting antigen; BGP I, biliary glycoprotein I; PS β G, pregnancy-specific β glycoprotein.


Immunoglobulin superfamily
N-CAM
CEA
Ig
MHC
TcR
etc
CEA-NCA Subfamily
PSβG Subfamily
?
CEA
NCA
BGP I
etc

architecture in adult and embryonic intestinal epithelium. In these studies, CEA mediates Ca^{2+}-independent, homotypic aggregation of cultured human colon adenocarcinoma cells transfected with functional CEA cDNA clones. The aggregation of CEA producing cells was completely inhibited by Fab' fragments of specific rabbit anti-CEA antibodies (Benchimol et al., 1989).

The existence of murine counterparts of CEA expressed in normal adult colon and liver has been identified. The sequence of a 726 bp partial murine cDNA clone isolated from colon of CD-1 adult mice was reported (Beauchemin et al., 1989a).

Antibody cross reactivity between human CEA and the MHV receptor was demonstrated in our laboratory (Williams et al., submitted for publication). This observation and the amino acid homology between the receptor and different human CEA family members were further explored by cloning and sequencing of the MHV receptor cDNA to determine the relationship between the CEA family of glycoproteins and the MHV receptor.

Results

Cloning of a partial cDNA coding for the MHV receptor : Three oligonucleotides, a sense primer, an antisense primer and a probe were designed based on the published partial murine CEA cDNA sequence (primers 10, 11 and 12 in Table 3).

RNA prepared from colon and liver of BALB/c mice was amplified with primers 10 and 11. The distance between the primers should yield a 490 bp product. As shown in figure 11, RNAPCR of colon and liver gave the expected size and two additional products. The three bands hybridized to the probe (12 in Table 3). The two smaller products could arise from other CEA family members not previously characterized in mouse that share this part of the sequence. To further verify the specificity of the products obtained, the annealing temperature was changed from 40° to 50°C. The same reaction products were detected on the gel and after probe hybridization (Figure 11 B). The 490 bp fragment was subcloned into the vector BlueScript SK+ (p490). Alignment of the predicted translation product for murine CEA with the human CEA protein sequence showed that the first 45 amino acids of the mature protein were missing in the published murine CEA cDNA clone (Figure 12).

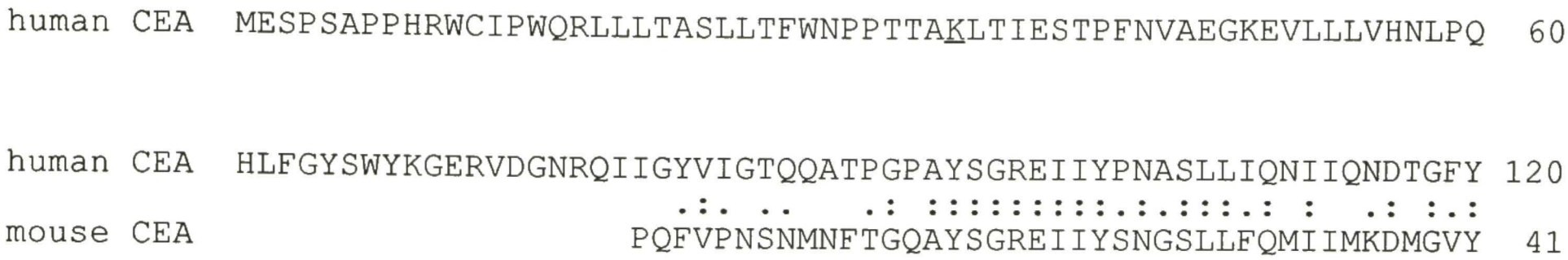
The N-terminal amino acid sequence for the MHV receptor available to us at that time, spanned the first 25 amino acids. We decided to explore the possibility that part of the murine 5' sequence absent in the reported partial murine CEA clone included the nucleotides coding for these 25 amino acids.

Figure 11. Effect of annealing temperature on production of the 490 bp RNAPCR product. Part A shows the relative amounts of the 3 bands produced by RNAPCR using primers 10 and 11 listed in Table 3. The RNA was isolated from liver (lane 1) and colon (lane 2) of BALB/c mice. The annealing temperature employed in the RNAPCR was 40°C. Part B shows the products obtained following amplification of BALB/c liver (lane 1) and colon RNA (lane 2) using an annealing temperature of 50°C. The autoradiograms of the Southern blots probed with the oligonucleotide 12 (Table 3) are shown.


B
1 2
A
1 2
490 bp

Figure 12. Alignment of the N-terminal domains of mCEA1 and human CEA as published by Beauchemin et al., 1989. The human and murine proteins have been aligned according to the BESTFIT program.

. indicates similarity between amino acids and : indicates identity. The underlined amino acid corresponds to the start of the mature human CEA protein.


human CEA MESPSAPPHRWCIPWQRLLLTASLLTFWNPPTTAKLTIESTPFNVAEGKEVLLLVHNLPQ 60
human CEA HLFGYSWYKGERVDGNRQIIGYVIGTQQATPGPAYSGREIIYPNASLLIQNIIQNDTGFY 120
mouse CEA PQFVPNSNMNFTGQAYSGREIIYSNGSLLFQMIIMKDMGVY 41

Primers 7, 8 and 9 designed based on the MHV receptorN-terminal protein sequence were employed to amplify cDNAs derived from tissue expressing the highest level of receptor protein (Williams et al., submitted for publication). The antisense primer and the probe corresponded to the partial CEA published sequence and were employed for the amplification of the 490 bp product described above. Colon RNA was reverse transcribed using primer 12 and the cDNA obtained was amplified in three separate PCR reactions using primers 7, 8 or 9. The predicted sizes for the products were detected in the three reactions. Figure 13 shows the 258 bp product obtained with primers 7 and 8 and antisense primer 12, and the 231 bp obtained with primers 9 and 12. Hybridization of the amplified material with oligonucleotide 10, verified the specificity of the reaction products (figure 13 B). The doublets seen upon probe hybridization resulted from the presence of single strand product accumulated following the exhaustion of the degenerate primers in the PCR reaction. Primer 9 was more effective in the amplification reaction than primers 7 and 8. Although successful amplification with oligonucleotides 7 and 8 with 12 was obtained several times, the product yields were very low and sometimes the amplified material was detectable only by probe hybridization. This is probably due to the difference in the degree of degeneracy of the oligonucleotides. Seven and 8 are 64-fold degenerate whereas 9 is 32-fold degenerate. Amplification of cDNAs derived from BALB/c small intestine RNA and poly A+ RNA from liver using primers 9 and 12 also yielded a product of 231 bp. Due to the low level of expression of this molecule in liver, poly A+ material had to be

Figure 13. Amplification of cDNA using degenerate amino terminal derived and murine CEA specific oligonucleotide primers. Total BALB/c colon RNA was reverse transcribed using primer 12 , then amplified by PCR using primer 12 paired with primer 7 (lane 1), primer 8 (lane 2) or primer 9 (lane 3). The reactions were cycled as described in the Methods section and 20 µl of each reaction was analyzed by electrophoresis on a 2 % agarose gel (part A) and the Southern blot probed with the sense oligonucleotide 10 (part B). Size markers confirm a 258 bp amplification product for lanes 1 and 2 and 231 bp product for lane 3. The doublets seen on probe hybridization result from the presence of single strand product accumulated following the exhaustion of the degenerate primers in the PCR reaction. The nucleotide sequences of the oligonucleotides employed are listed in Table 3.

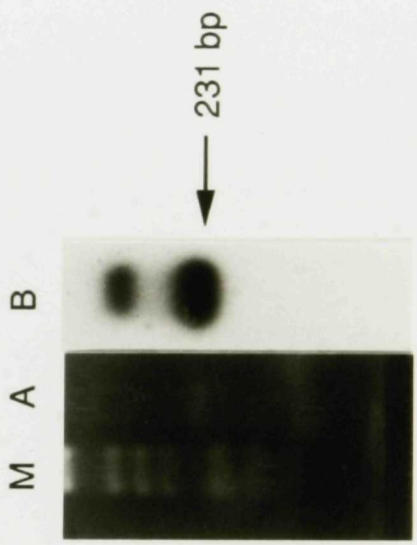

A
1 2 3
B
1 2 3
258 bp
231 bp

employed for successful amplification (Figure 14).

Subcloning of the 258 bp colon derived RNAPCR- fragment was attempted several times without success. The colon derived 231 bp fragment was subcloned into the pUC 18 vector and sequenced. Comparison of this PCR-derived fragment to the published partial cDNA clone of mouse CEA-related glycoprotein revealed two nucleotide differences within the first two amino acids of the published clone. The two base changes were detected in independent clones of this region and confirmed after sequencing both strands. These differences are of importance because they represent non conservative changes in the amino acids for these codons. An alanine corresponding to amino acid 46 of the receptor substitutes for a proline in the published sequence and the arginine at position 47 replaces the glutamine. Differences with the published partial murine cDNA sequence could represent allelic forms of this gene.

Since the goal was to clone the full length cDNA coding for the MHV receptor we needed to obtain a larger fragment suitable for library screening. Although the 231 bp fragment could confer the specificity needed, its length is too short for optimal radiolabeling. For the purpose of obtaining a longer probe, colon RNA was amplified using an antisense primer based on the 3' end of the reported CEA sequence and the sense primer 9. This antisense primer (number 13 in Table 3), was designed to contain a Sal I recognition site to facilitate subcloning. The product of the amplification reaction was 710 bp as predicted. It was subcloned into BlueScript SK+ and named p710. Various clones were

Figure 14. Production of the 231 bp product from poly A+ liver RNA. RNAPCR was performed using primers 9 and 12 (listed in Table 3), starting with 0.5 µg of poly A+ liver RNA isolated from BALB/c mice. Following amplification for 25 cycles, 10% of the reaction was electrophoresed. A photograph of the ethidium bromide stained gel is shown in part A. Lane M corresponds to the 1 kb molecular size marker (Life Technologies). An autoradiogram of the Southern blot of the agarose gel from A using oligonucleotide 10 (Table 3) as a probe is shown in part B. The doublets seen here arose from the use of degenerate oligonucleotide primers as also seen in Figure 13.


231 bp
M
A
B

obtained and sequenced. Because a degenerate oligonucleotide was employed for amplification, the clones analyzed differ in their sequence corresponding to the third position of the amino acids selected to be covered by more than one possible codon. The nucleotide difference is the result of the ability of more than one oligonucleotide in the pool to bind to the template and prime the amplification reaction. Although all the clones had the capability to be translated to the receptor amino acids, we could not determine which of the p710 clones truly represented the nucleotide sequence corresponding to the actual receptor cDNA. The rest of the nucleotide sequence of p710 was identical to that of p231 and continued in the same open reading frame to include the sequence of the partial murine CEA related cDNA clone (Beauchemin et al., 1989a).

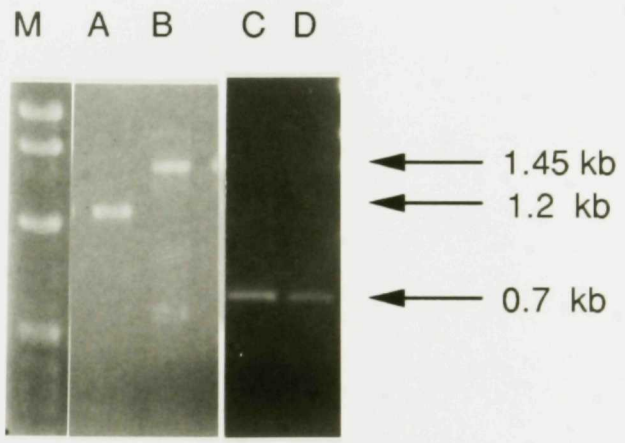
A BALB/c liver gt11 cDNA library (10^9 pfu/ml) (Promega), a JLSV9 cDNA library (10^7 pfu/ml) (constructed by Dr Dieffenbach) and a mouse NIH 3T3 cosmid library in pWE15 (10^9 pfu/ml) (Stratagene) were screened with the 710 bp fragment described above. Six hundred thousand plaques were screened from the JLSV9 and the BALB/c liver library and 120,000 colonies were screened from the cosmid library with the nick translated fragment. Duplicate filters were analyzed in each case. The first round of screening resulted in no positive plaques from the JLSV9 library and four positive plaques from the lambda gt11 BALB/c liver cDNA library. No positive colonies were identified from the cosmid library. For isolation of the positive phage inserts from the BALB/c liver library, a second round of screening was performed. Two of the

original positive plaques did not hybridize to the probe and the other two were further plated and screened a third time. After the identification of single isolated positive plaques, the insert size was determined by amplification of the phage DNA with the lambda forward and reverse sequencing primers (New England Biolabs, 1218 and 1222) followed by electrophoresis of the product on agarose. The two cDNA clones obtained were designated MHVR1.4 and MHVR1.1. An additional second round screening with a pool of end labelled oligonucleotides (10, 14, 21, 22, 23 in Table 3) recovered the two additional positive signals from the BALB/c liver library. Single plaques were isolated after two additional rounds of screening. The insert sizes were determined by PCR amplification of the phage DNA as described above. The sizes are 1.45 kb and 2.9 kb. Sequencing of these two clones is in progress.

MHVR1.4 is 1,389 bp long and MHVR1.1 is 1,039 bp long. To establish the possible relationship of the two phage clones containing different size inserts, phage DNA was amplified with primer 14 derived from the 710 bp fragment and the lambda gt11 reverse sequencing primer. The two clones yielded the same size product, indicating 3' co-linearity (Figure 15). Amplified products for both clones hybridized to labeled oligonucleotide 12.

MHVR1.4 and MHVR1.1 were subcloned into the EcoR I-Not I sites of BlueScript SK+. Two clones from MHV1.4 were obtained. They are referred as pMHVR1 and pMHVR2. The clone obtained from the smaller phage insert, MHVR1.1 is pMHVR3. Eleven oligonucleotides were employed in the sequencing of the two strands of each clone. The sequence of pMHVR2 is

Figure 15 . Determination of insert size and 3'-colinearity between the two MHV receptor clones. Phage plaques producing a positive hybridization signal with p710 were plucked and eluted as described in the Methods section. The eluted material was amplified with lambda gt11 forward and reverse primers (New England Biolabs), lanes A and B, and lambda gt11 reverse and primer 14 (Table 13) in lanes C and D. Lane M corresponds to the 1 kb molecular size marker (Life Technologies). A portion of the reaction was electrophoresed and a photograph of the ethidium bromide stained 1.5 % agarose gel is shown.


M
A
B
C
D
1.45 kb
1.2 kb
0.7 kb

shown in figure 16. pMHVR1 and pMHVR2 start at the third position of codon 9 of the affinity purified receptor protein and extend to the poly A tail. The two nucleotide differences previously observed when comparing the sequences of p231 and p710 to the published 726 bp sequence of the murine cDNA clone of CEA were also present in pMHVR1 and pMHVR2 (Beauchemin et al.,1989). The nucleotide at position 468 of pMHVR1 was also found to differ from the published 726 bp sequence. This difference reflects the substitution of an asparagine for an isoleucine and the loss of a potential N-linked glycosylation site. The sequence of pMHVR2 is identical to the published sequence in that position. We think that the nucleotide difference between pMHVR1 and pMHVR2 is the result of an error during amplification. Taq polymerase has no proof-reading activity resulting in an error rate of 1 in 80,000 bases (Tindall and Kunkel, 1988). This observation shows the need to sequence more than one clone when analyzing PCR products of sequences not previously reported. This problem can be circumvented by directly sequencing the PCR products avoiding the subcloning step if there is no need to have the amplified material in a vector for further experiments. Errors introduced during polymerization are irrelevant to the sequence determination since each individual misincorporated base will be represented only very infrequently in the population of DNA molecules to be sequenced (Reiss et al., 1990). The sequence of pMHVR3 starts at position 350 of pMHVR2 and extends to the poly A tail. Identity of the 3' end of the two lambda clones obtained from the same library indicates that the nucleotide sequence of MHVR1.1 is contained in MHVR1.4. Figure 17 shows

Figure 16. Nucleotide sequence of pMHVR2.

CCAGGTTGCT	GAAGACAACA	ATGTTCTTCT	ACTTGTTCAC	AATCTGCCCC	TGGCGCTTGG
" 10	" 20	" 30	" 40	" 50	" 60
AGCCTTTGCC	TGGTACAAGG	GAAACACTAC	GGCTATAGAC	AAAGAAATTG	CACGATTTGT
" 70	" 80	" 90	" 100	" 110	" 120
ACCAAATAGT	AATATGAATT	TCACGGGGCA	AGCATACAGC	GGCAGAGAGA	TAATATACAG
" 130	" 140	" 150	" 160	" 170	" 180
CAATGGATCC	CTGCTCTTCC	AAATGATCAC	CATGAAGGAT	ATGGGAGTCT	ACACACTAGA
" 190	" 200	" 210	" 220	" 230	" 240
TATGACAGAT	GAAAACTATC	GTCGTACTCA	GGCGACTGTG	CGATTTCATG	TACACCCCAT
" 250	" 260	" 270	" 280	" 290	" 300
ATTATTAAAG	CCCAACATCA	CAAGCAACAA	CTCCAATCCC	GTGGAGGGTG	ACGACTCCGT
" 310	" 320	" 330	" 340	" 350	" 360
ATCATTAACC	TGTGACTCTT	ACACTGACCC	TGATAATATA	AACTACCTGT	GGAGCAGAAA
" 370	" 380	" 390	" 400	" 410	" 420
TGGTGAAAGC	CTTTCAGAAG	GTGACAGGCT	GAAGCTGTCT	GAGGGCAACA	GGACTCTCAC
" 430	" 440	" 450	" 460	" 470	" 480
TTTACTCAAT	GTCACGAGGA	ATGACACAGG	ACCCTATGTG	TGTGAAACCC	GGAATCCAGT
" 490	" 500	" 510	" 520	" 530	" 540
GAGTGTCAAC	CGAAGTGACC	CATTCAGCCT	GAACATTATC	TATGGTCCGG	ACACCCCGAT
" 550	" 560	" 570	" 580	" 590	" 600
TATATCCCCC	TCAGATATTT	ATTTGCATCC	AGGGTCAAAC	CTCAACCTCT	CCTGCCATGC
" 610	" 620	" 630	" 640	" 650	" 660
AGCCTCTAAC	CCACCTGCAC	AGTACTTTTG	GCTTATCAAT	GAGAAGCCCC	ATGCATCCTC
" 670	" 680	" 690	" 700	" 710	" 720

CCAAGAGCTC TTTATCCCCA ACATCACTAC TAATAATAGC GGAACCTATA CCTGCTTCGT
" 730 " 740 " 750 " 760 " 770 " 780

CAATAACTCT GTCACTGGCC TCAGTAGGAC CACAGTCAAG AACATTACAG TCCTTGAGCC
" 790 " 800 " 810 " 820 " 830 " 840

AGTGACTCAG CCCTTCCTCC AAGTCACCAA CACCACAGTC AAAGAACTAG ACTCTGTGAC
" 850 " 860 " 870 " 880 " 890 " 900

CCTGACCTGC TTGTCGAATG ACATTGGAGC CAACATCCAG TGGCTCTTCA ATAGCCAGAG
" 910 " 920 " 930 " 940 " 950 " 960

TCTTCAGCTC ACAGAGAGAA TGACACTCTC CCAGAACAAC AGCATCCTCA GAATAGACCC
" 970 " 980 " 990 " 1000 " 1010 " 1020

TATTAAGAGG GAAGATGCCG GCGAGTATCA GTGTGAAATC TCGAATCCAG TCAGCGTCAG
" 1030 " 1040 " 1050 " 1060 " 1070 " 1080

GAGGAGCAAC TCAATCAAGC TGGACATAAT ATTTGACCCA ACACAAGGAG GCCTCTCAGA
" 1090 " 1100 " 1110 " 1120 " 1130 " 1140

TGGCGCCATT GCTGGCATCG TGATTGGAGT TGTGGCTGGG GTGGCTCTAA TAGCAGGGCT
" 1150 " 1160 " 1170 " 1180 " 1190 " 1200

GGCATATTTC CTCTATTCCA GGAAGTCTGG CGGATCTGGC TCCTTCTGAC AACTCTCCTA
" 1210 " 1220 " 1230 " 1240 " 1250 " 1260

ACAAGGTGGA TGACGTCGCA TACACTGTCC TGAACTTCAA TTCCCAGCAA CCCAACCGGC
" 1270 " 1280 " 1290 " 1300 " 1310 " 1320

CAACTTCAGC CCCTTCTTCT CCAAGAGCCA CAGAAACAGT TTATTCAGAA GTAAAAAAGA
" 1330 " 1340 " 1350 " 1360 " 1370 " 1380

AGTGAGCATA AAAAAAAAAA AAAAAAAAAA AA
" 1390 " 1400 " 1410 " 1420

Figure 17. Amino acid sequence of the MHV receptor predicted from the nucleotide sequence of clone pMHVR2. The one letter amino acid code is used. The * indicates the presence of a stop codon. The potential N-linked glycosylation sites (Asn-X-Ser /Thr) are underlined and cysteines are indicated in bold.

1 EVTIEAVPPQVAEDNNVLLLVHNLPLALGAFAWYKGNTTAIDKEIARFVP

51 NSNMNFTGQAYSGREIIYSNGSLLFQMITMKDMGVYTLDMTDENYRRTQA

101 TVRFHVHPILLKPNITSNNSNPVEGDDSVSLTCDSYTDPDNINYLWSRNG

151 ESLSEGDRLKLSEGNRTLTLLNVTRNDTGPYVCETRNPVSVNRSDPFSLN

201 IIYGPDTPIISPSDIYLHPGSNLNLSCHAASNPPAQYFWLINEKPHASSQ

251 ELFIPNITTNNSGTYTCFVNNSVTGLSRTTVKNITVLEPVTQPFLQVTNT

301 TVKELDSVTLTCLSNDIGANIQWLFNSQSLQLTERMTLSQNNSILRIDPI

351 KREDAGEYQCEISNPVSVRRSNSIKLDIIFDPTQGGLSDGAIAGIVIGVV

401 AGVALIAGLAYFLYSRKSGGSGSF*

the amino acid sequence coded for pMHVR2.

Characterization of the transcript coding for the MHV receptor : To determine the size of the transcript that codes for the MHV receptor, Northern blot hybridization was performed on total RNA prepared from BALB/c and SJL/J mice colon and small intestine. Two different hybridization probes were employed. One is the insert of p710 and the other is the insert of pMHVR1. After washes using stringent conditions, a 3.4 kb transcript was detected in both tissues with the p710 insert after a two day exposure. When the blot was probed with the insert of pMHVR1 and exposed for a week, a second transcript of 1.7 kb was also detected in both tissues (Figure 18). These results agree with the reports of Beauchemin et al. (Beauchemin, 1989a). They observed an abundant 3.8-3.4 kb transcript in adult colon and less abundant shorter transcripts in colon and liver tissues. These transcripts probably correspond to other membranes of the murine CEA gene family. Different murine cDNA clones specific for shorter transcripts have been isolated from a CD-1 mice colonic cDNA library, although their sequences have not been reported (Beauchemin, 1989b).

A 5-10 kDa size difference has been observed for the MHV receptor protein found in BALB/c mice and its protein homolog detected with a polyclonal antibody directed to the first 15 amino acids of the receptor in the resistant SJL/J mice (Williams et al., 1990). The size difference was not reflected in the transcripts derived from the two mouse strains. The pattern of hybridization shown in Figure 19 shows that the degree of similarity present between the

Figure 18. Northern blot analysis of RNA prepared from BALB/c colon and small intestine. Ten micrograms of total RNA from colon (lanes A and A*) and small intestine (lanes B and B*) were analyzed by Northern blot hybridization; the probes employed were the insert of p710 for lanes A and B and the insert of pMHVR1 for lanes A* and B*. The sizes of the transcripts were calculated in relationship to the 28s and 18s ribosomal bands and an RNA marker. Lanes A and B represent hybridization signals from an overnight exposure, and A* and B* from one week long exposure.

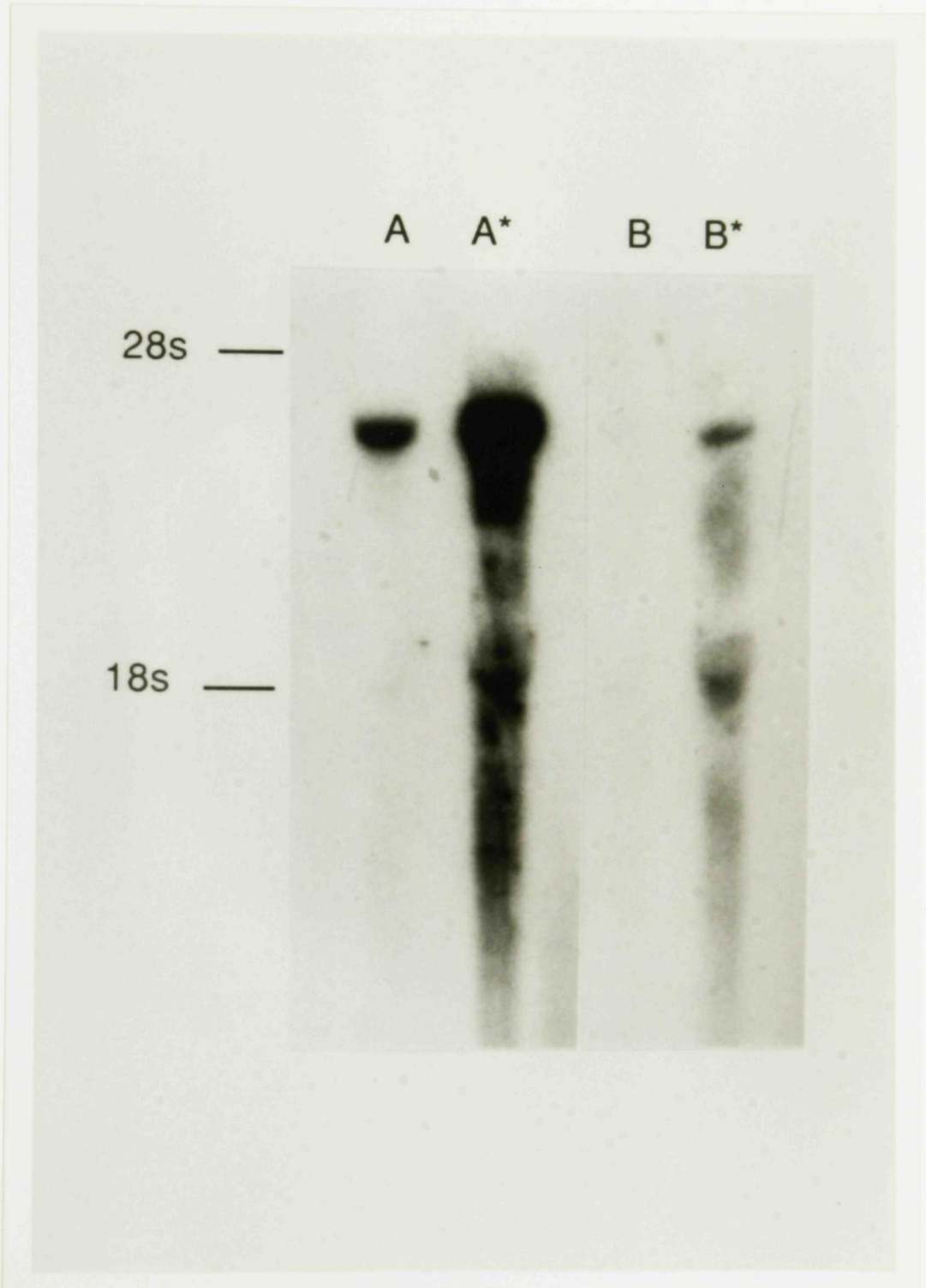
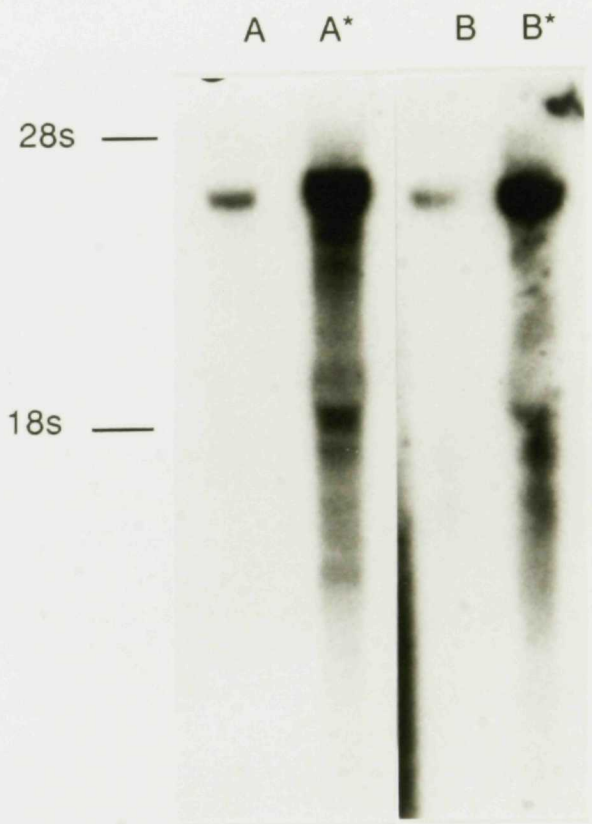

A
A*
B
B*
28s
18s

Figure 19. Northern blot analysis of RNA prepared from BALB/c and SJL/J colon. Ten micrograms of total RNA from colon of BALB/c mice (lanes A and A*) and colon of SJL/J mice (lanes B and B*) were analyzed by Northern blot hybridization; the probe employed was the insert of p710 for lanes A and B and the insert of pMHVR1 for lanes A* and B*. The sizes of the transcripts were calculated in relationship to the 28s and 18s ribosomal bands and an RNA marker. Autoradiographic exposures of overnight (lanes A and B) and one week (lanes A* and B*) are shown.


A
A*
B
B*
28s
18s

message coding for the functional MHV receptor in BALB/c mice and the message in SJL/J represents more than the first 15 amino acids at the protein level. Because the level of resolution in a Northern blot does not permit a fine determination of the size of the transcript, we decided to explore the possibility of a size difference between BALB/c and SJL/J by RNAPCR of the first half of the molecules. The sizes of the products obtained with primers 10 and 11, and 9 and 11 did not show a detectable difference (Figure 20). These products hybridized to the specific probes.

Genomic DNA isolated from liver of BALB/c and SJL/J mice was digested with the restriction enzymes EcoR I and Sst I. Southern blot hybridization to the insert of pMHVR1 was performed. In an attempt to determine the number of copies of this gene, different amounts of linearized pMHVR1 corresponding to 0.1,1 and 10 copies per mouse genome were electrophoresed in the same agarose gel as the genomic samples. Comparable amounts of empty vector were also analyzed in this Southern blot as a negative control. Two restriction fragment length polymorphisms were detected as shown in figure 21. The series of bands with roughly equivalent hybridization intensities most likely represent bands arising from closely related gene or genes and the bands producing a faint background almost certainly arise from cross hybridization between the probe and other more distantly related CEA family members. The difference at the DNA level between these two mice strains is reflected by the hybridization pattern obtained after restriction of the DNA with these two enzymes. These differences could map to an intron or exon, to the coding or

Figure 20. Determination of RNAPCR product sizes from BALB/c and SJL/J colon and small intestine RNA. RNAPCR for the comparison of the MHV receptor transcript from BALB/c and SJL/J mice was performed. Part A shows the photograph of the ethidium bromide stained gel and part B shows the autoradiogram of the Southern blot of the same gel. The 490 bp product was obtained using primers 10 and 11 with SJL/J colon (lane 1) and BALB/c colon (lane 2) RNA. For the 231 bp primers 9 and 11 were employed with SJL/J small intestine (lane 3), BALB/c small intestine (lane 4), SJL/J colon (lane 5) and BALB/c colon (lane 6) RNA. As shown in part B, the 490 bp was detected with end labeled oligonucleotide 12 and the 231 bp product was detected with end labeled oligonucleotide 10. The sequences of the oligonucleotides employed are listed in Table 3.

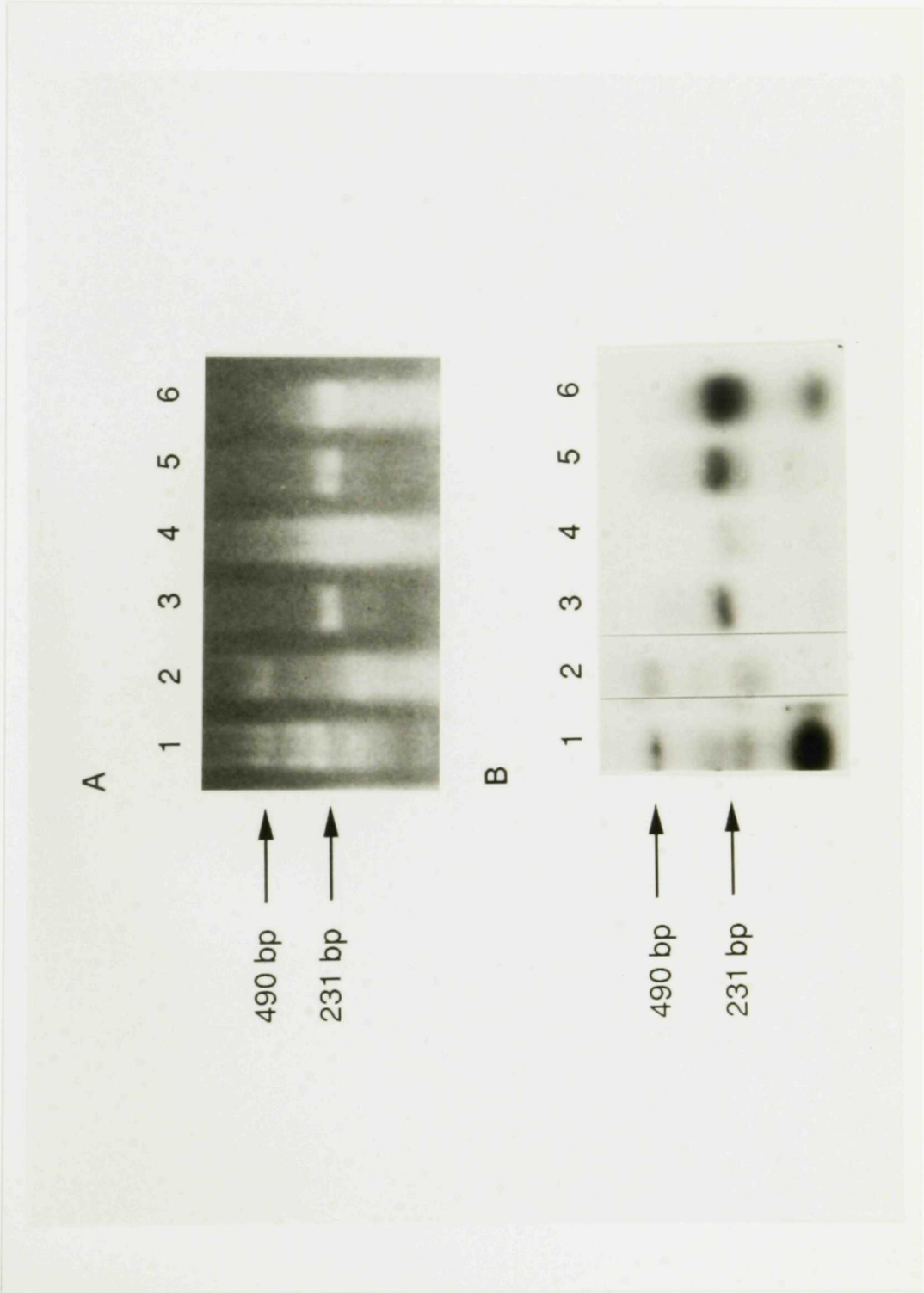
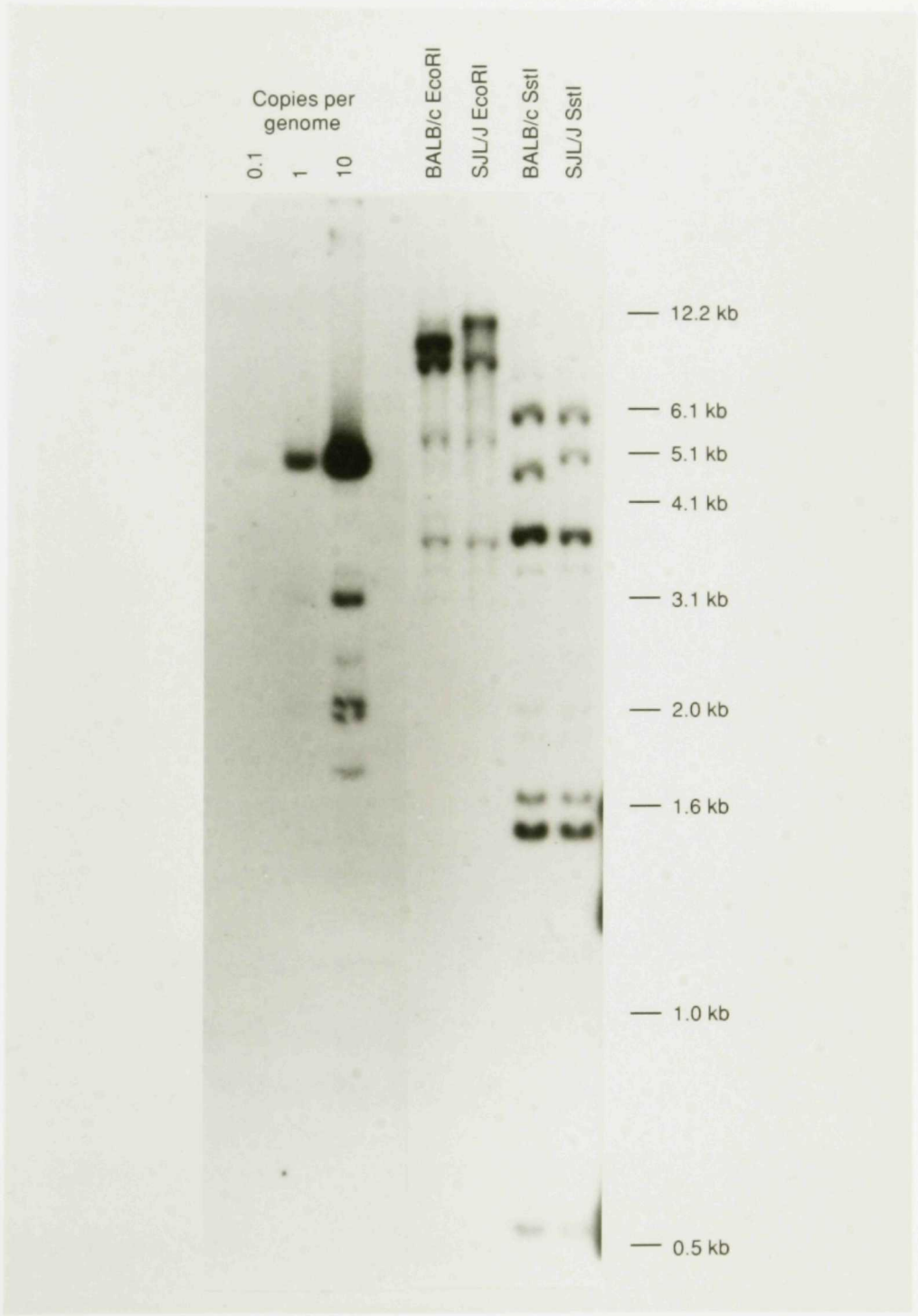

A
1 2 3 4 5 6
490 bp
231 bp
B
1 2 3 4 5 6
490 bp
231 bp

Figure 21. Analysis of liver genomic DNA of BALB/c and SJL/J mice. BALB/c and SJL/J liver DNA was digested with Eco RI and Sst I and 10 μg of each was electrophoresed on a 0.8 % agarose gel. Following Southern blotting, the nytran membrane was hybridized to pMHVR1 as described in the Methods section. The blot was washed with 0.1 % SSPE, 0.1 % SDS at 55°C for 90 minutes. Linearized pMHVR1 corresponding to 0.1, 1 and 10 copies per genome is also shown.


Copies per genome
0.1
1
10
BALB/c EcoRI
SJL/J EcoRI
BALB/c SstI
SJL/J SstI
12.2 kb
6.1 kb
5.1 kb
4.1 kb
3.1 kb
2.0 kb
1.6 kb
1.0 kb
0.5 kb

non-coding region of the transcript, and could be the result of one or more point mutations, deletions or insertions. The cloning of the homolog cDNA in the SJL/J mice strain in progress in our laboratory will answer this question.

Cloning of the 5' end of the transcript coding for the MHV receptor : The PCR-derived p213 and p710 clones started at amino acid number 10. Clones pMHVR1 and pMHVR2 obtained from the library started at the third position of amino acid 9 of the mature receptor protein. Different approaches were attempted to obtain the 5' end of the MHV receptor transcript using RNA as starting material. One of them is illustrated in figure 2. Two antisense primers derived from the 5' end of MHVR1.4 were employed (primers 15 and 16 in Table 3). The most 3' of the primers contains a Sal I site to facilitate further subcloning of the obtained product. The reverse transcriptase reaction was primed with oligonucleotide 15 and primers 5, 6 and 16 were employed for the first amplification of the cDNA after tailing the cDNA with dATP as described in the Methods section. A small portion of the amplified material was re-amplified using the same primers. Six of these reamplification reactions were pooled for subcloning into the Not I - EcoR V sites of BlueScript SK+. One 230 bp clone was obtained and sequenced. The sequence of primer 15 was present followed by 29 nucleotides which correspond to the first 9 amino acids of the receptor and an alanine corresponding to the last amino acid of the leader peptide sequence. After the nucleotides coding for the alanine, twenty adenosines were observed. The sequence of the sense primer was not present and the fragment was not inserted in the Not I site as expected. This indicated the possibility of

the presence of an internal Not I site in the 5' end of the receptor sequence. Since the sense primer employed was designed to contain both a Not I recognition site and a Sst II site, we attempted the subcloning of the amplified material in the Sst II - EcoR V sites of BlueScript SK+ and the pUC18 vectors. Three different clones were obtained and sequenced. The sequence of the sense primer was not present and different rearrangments of the plasmid were observed. One of these three clones contained the sequence for the first 6 codons of the mature receptor protein followed by plasmid sequence.

The leader peptides of six different CEA family members, including the biliary glycoprotein I, were compared by Khan et al., (1989). There is a high degree of homology between the leader peptides at the amino acid level: they are 34 amino acids long and contain a conserved alanine at position -1. The amino acid sequence derived from MHVR1.4 showed that the most closely related CEA human family member is the biliary glycoprotein as shown bellow. A 4-fold degenerate oligonucleotide primer derived from the first 7 amino acids of the human biliary glycoprotein leader peptide was designed (Primer 17 in Table 3) for RNAPCR amplification of the 5' coding region of the MHV receptor. A Kpn I recognition site was added at the 5' end of the oligonucleotide to facilitate subcloning. This restriction enzyme recognition sequence was selected because it also provides a good context for initiating translation according to the Kozak rules (Kozak,1989). Colon and small intestine BALB/c RNA were reverse transcribed with primer 15, and the cDNA was amplified with primers 15 and 17. The expected product is 188 bp long, assuming

conservation of the length of the leader peptide. Although the product of the reactions was not a distinct band of the expected size when analyzed by agarose gel electrophoresis, specific hybridization to oligonucleotide 16 aws detected (data not shown). The reaction product was digested with Kpn I and Sal I and subcloned into BlueScript SK+. Although the two primer sequences were present at the correct cloning sites, the sequence of the primer corresponding to the leader peptide followed that of primer 15 in place of the expected sequence corresponding to primer 16.

Because of the various rearrangments observed, we decided to attempt the amplification of the 5' end of the receptor using BALB/c liver DNA as the starting material (Figure 1). After digestion of the DNA with Mbo I, BamH I, Xho II and Bgl II, the DNA was ligated to the oligonucleotides 18 and 19 in Table 3. These adaptor-ligated DNAs were amplified with primers 20 and 15. Although no product was detected in any of the amplification reactions, reamplification with primers 20 and 16 of the Mbo I digested DNA yielded a 180 bp fragment as shown in figure 22. This PCR product was subcloned into the BamH I-Hinc II sites of pUC18 and sequenced. This clone is designated p90. The sequence obtained was compared with rat CEA related glycoprotein (Lin and Guidotti, 1989) and showed that 90 nucleotides most certainly corresponded to the first 15 amino acids of the leader peptide and amino acids 1 to 15 of the mature protein. The 5' end of the cloned sequence corresponds to an intron (Figure 23). The first 10 amino acids derived from the translation of this genomic sequence agree with the protein sequence obtained from the affinity purified

Figure 22. PCR product obtained by amplification of Mbo I digested genomic BALB/c DNA. A picture of the ethidium bromide stained polyacrylamide gel shows the 180 bp band obtained upon reamplification of the PCR reaction using primers 20 and 15 (Table 3) as described in the Methods section. Lane M corresponds to the 123 bp ladder (Life Technologies).

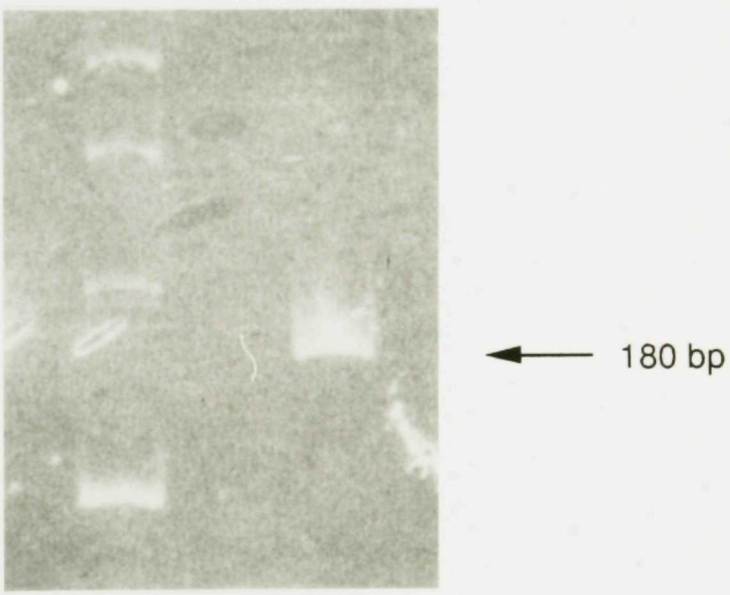

M
180 bp

Figure 23. Alignment of the genomic DNA-derived PCR product with the rat ecto-ATPase. Two dots indicate identity between the two sequences. The consensus splice acceptor site is underlined (Shapiro and Senapathy, 1987).

```
MHV receptor     TCCTAAGATTGATAGGTCTTTCTCTCTTCCCTCTTAGCCTCACTTTTAGCCTCC
                    :::   :::::::::::::::  ::: :
rat ecto-ATPase  AGAGGGCAGATTCCCTGGAGGGGACTACTGCTCACAGCCTCACTTTTAACCTAC

TGGAGCCCTGCCACCACTGCTGAAGTCACCATTGAGGCTGTCGGCGCCC-AGGTTGCTGAAGACAAC
:::::::::  ::::::::: : :: ::::: : ::: : :::: ::: :: ::
TGGAGCCCTCTCACCACTGCCCAAGTCACCGTAGACGGCTGTGCCACCCAACGTTGTTGAGGAGAAG
```

receptor and previously observed in the rearranged 230 bp clone described above.

The relative positions of the different clones obtained for the MHV receptor are shown in figure 24.

Structure of the receptor protein : Comparison of the protein sequences and domain structure of CEA related glycoproteins showed that the closest human homolog to the MHV receptor is the biliary glycoprotein I (Hinoda et al., 1988). The amino acid identity between these two molecules is 54% and the amino acid similarity 66% (Figure 25). Human biliary glycoprotein I shows a high degree of homology with the rat ecto-ATPase (Lin and Guidotti, 1989). The homology of the rat ecto-ATPase with the MHV receptor is 67% identity and 77% similarity at the amino acid level (Figure 26). The positions of the cysteines are conserved in these 3 glycoproteins.

As shown in figure 27, the putative extracellular region of the MHV receptor glycoprotein could form 3 disulfide linked loops like those of immunoglobulin constant domains and the first loop is defined by a salt bridge formed by the arginine and aspartic acid residues (Williams, 1987). Near the carboxyl terminus, a potential transmembrane domain was identified by hydrophilicity analysis according to both the Kyte-Doolittle and the Hopp-Woods method (Figure 28). This protein presents sixteen potential N-linked glycosylation sites (Asn -X -Thr or Ser).

In vitro transcription and translation of a partial receptor clone : Previous results suggested that the first 15 amino acids of the MHV receptor are not

Figure 24. Relative positions of the different MHV receptor clones obtained. PCR products are indicated by *, the partial murine CEA published sequence (Beauchemin et al.,1989) by **, and the clones isolated from a BALB/c liver cDNA library by ***. The first amino acid of the mature receptor protein is denoted as +1.

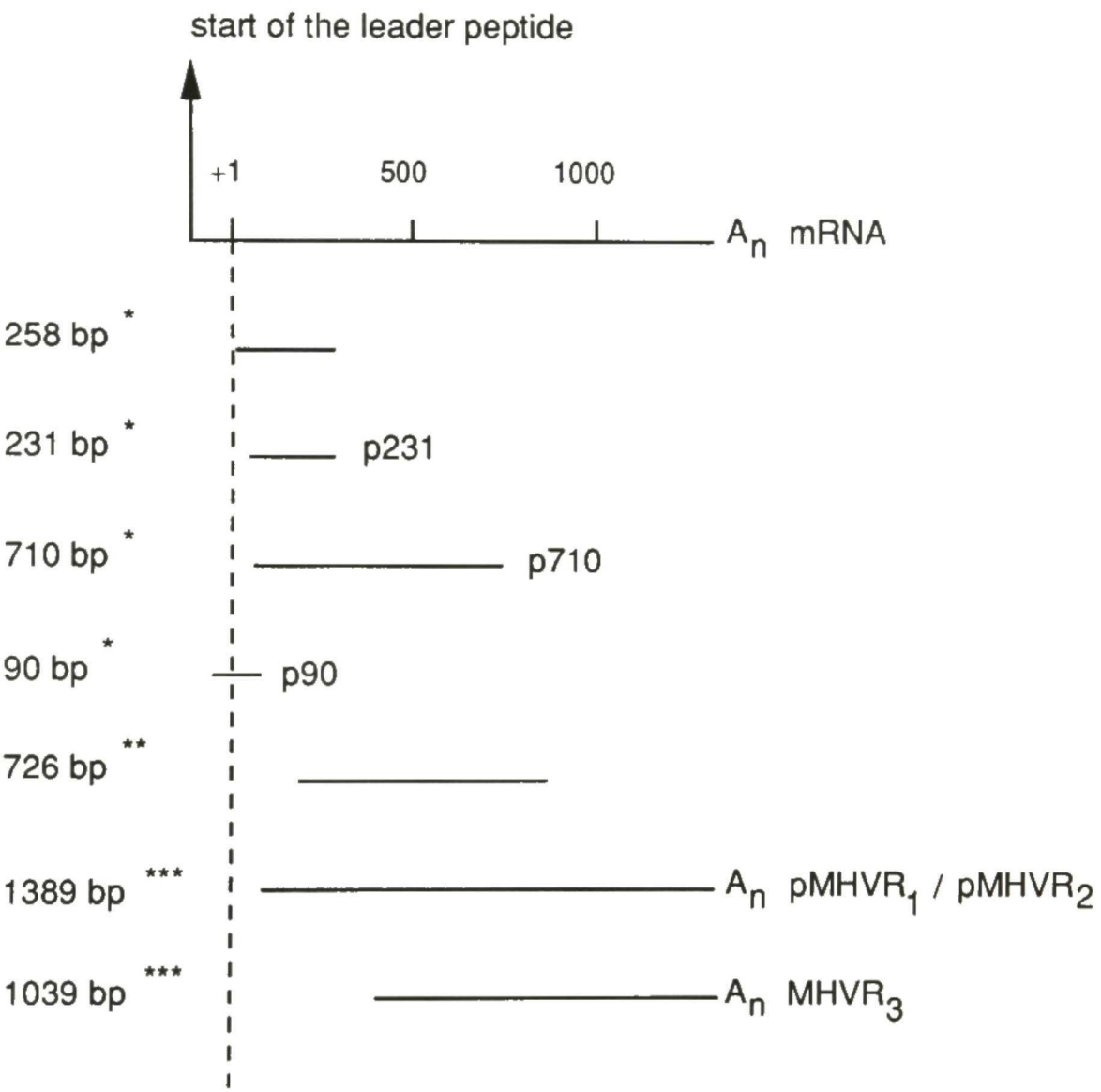

start of the leader peptide
+1
500
1000
A_n mRNA
258 bp *
231 bp *
p231
710 bp *
p710
90 bp *
p90
726 bp **
1389 bp ***
A_n $pMHVR_1$ / $pMHVR_2$
1039 bp ***
A_n $MHVR_3$

Figure 25. Alignment of the predicted amino acid sequence of the human biliary glycoprotein I (BGP I) and the MHV receptor. The amino acid sequences of the MHV receptor and BGP I were compared using the BESTFIT program. Two dots indicates similarity between the amino acids and a bar indicates identity between amino acids.

```
  1 EVTIEAVPPQVAEDNNVLLLVHNLPLALGAFAWYKGNTTAIDKEIARFVP 50
    ::|.|.:| .|||:.:|||||||||  | ::.||||: .. :::|. :.
 62 QLTTESMPFNVAEGKEVLLLVHNLPQQLFGYSWYKGERVDGNRQIVGYAI 111

 51 NSNMNFTGQAYSGREIIYSNGSLLFQMITMKDMGVYTLDMTDENYRRTQA 100
    ... . .|.| ||||.||.|:|||:| :| .| |.|||::...:. ..:|
112 GTQQATPGPANSGRETIYPNASLLIQNVTQNDTGFYTLQVIKSDLVNEEA 161

101 TVRFHVHPILLKPNITSNNSNPVEGDDSVSLTCDSYTDPDNINYLWSRNG 150
    |..|||.| | ||.|.|||||||:.|.|.:||:.  :.:|:..|||. |.
162 TGQFHVYPELPKPSISSNNSNPVEDKDAVAFTCEP..ETQDTTYLWWINN 209

151 ESLSEGDRLKLSEGNRTLTLLNVTRNDTGPYVCETRNPVSVNRSDPFSLN 200
    :||. :.||.||:||||||||.|||||||| ||..||||.|||||..||
210 QSLPVSPRLQLSNGNRTLTLLSVTRNDTGPYECEIQNPVSANRSDPVTLN 259

201 IIYGPDTPIISPSDIYLHPGSNLNLSCHAASNPPAQYFWLINEKPHASSQ 250
    :.||||||.|||||.|.:||.||.|||.|||||||| ||||:. :.|.|
260 VTYGPDTPTISPSDTYYRPGANLSLSCYAASNPPAQYSWLINGTFQQSTQ 309

251 ELFIPNITTNNSGTYTCFVNNSVTGLSRTTVKNITVLE...PVTQPFLQV 297
    |||||||||.||||.||| .|||||| .|||||.|.| |   .|..| :..
310 ELFIPNITVNNSGSYTCHANNSVTGCNRTTVKTIIVTELSPVVAKPQIKA 359

298 TNTTVK.ELDSVTLTCLSNDIGANIQWLFNSQSLQLTERMTLSQNNSILR 346
    ..|||. : |||.||| .||.| .|.|:|..|||. .|||.|||.|..|.
360 SKTTVTGDKDSVNLTCSTNDTGISIRWFFKNQSLPSSERMKLSQGNTTLS 409

347 IDPIKREDAGEYQCEISNPVSVRRSNSIKLDIIFD..PTQGGLSDGAIAG 394
    |:|:|||||||.| ||: ||:| ..|:.|.|:: ::  | :.|||.|||||
410 INPVKREDAGTYWCEVFNPISKNQSDPIMLNVNYNALPQENGLSPGAIAG 459

395 IVIGVVAGVALIA.GLAYFLYSRKSGGSGS 423
    ||||||| ||||| :||:||.  |.| .:.
460 IVIGVVALVALIAVALACFLHFGKTGRASD 489
```

Figure 26. Alignment of the predicted amino acid sequences of the rat ecto-ATPase and the MHV receptor. The amino acid sequences of the MHV receptor and the rat ecto-ATPase were compared using the BESTFIT program. Two dots indicates similarity between the amino acids and a bar indicates identity between amino acids.

```
  1 EVTIEAVPPQVAEDNNVLLLVHNLPLALGAFAWYKGNTTAIDKEIARFVP 50
    :||::||||.|.|:..||||.||||  :..| ||||.| . |.||||::.
 56 QVTVDAVPPNVVEEKSVLLLAHNLPQEFQVFYWYKGTTLNPDSEIARYIR 105

 51 NSNMNFTGQAYSGREIIYSNGSLLFQMITMKDMGVYTLDMTDENYRRTQA 100
    ..||. ||.||||||.|||||||:|| :...|  .|||.: |:.:...|.
106 SDNMSKTGPAYSGRETIYSNGSLFFQNVNKTDERAYTLSVFDQQFNPIQT 155

101 TVRFHVHPILLKPNITSNNSNPVEGDDSVSLTCDSYTDPDNINYLWSRNG 150
    .|.|:|.| | |||:|:|||||:||:. ||| |:.|| :|..|||||||
156 SVQFRVYPALQKPNVTGNNSNPMEGEPFVSLMCEPYT..NNTSYLWSRNG 203

151 ESLSEGDRLKLSEGNRTLTLLNVTRNDTGPYVCETRNPVSVNRSDPFSLN 200
    ||||||||:.:|||||||||||| |.|.| | ||.|||...||||||.|:
204 ESLSEGDRVTFSEGNRTLTLLNVRRTDKGYYECEARNPATFNRSDPFNLD 253

201 IIYGPDTPIISPSDIYLHPGSNLNLSCHAASNPPAQYFWLINEKPHASSQ 250
    :|||||.|:|||.|||||.||||||||||.|||||||||||| :.|||
254 VIYGPDAPVISPPDIYLHQGSNLNLSCHADSNPPAQYFWLINEKLQTSSQ 303

251 ELFIPNITTNNSGTYTCFVNNSVTGLSRTTVKNITVLEPVTQPFLQVTNT 300
    ||||.||||||||||.|||||.|||||||||||||||:|||||| :|:|||
304 ELFISNITTNNSGTYACFVNNTVTGLSRTTVKNITVFEPVTQPSIQITNT 353

301 TVKELDSVTLTCLSNDIGANIQWLFNSQSLQLTERMTLSQNNSILRIDPI 350
    |||||:||||||:|.|.|..:.||||||||||:||||||:||.||||||
354 TVKELGSVTLTCFSKDTGVSVRWLFNSQSLQLTDRMTLSQDNSTLRIDPI 403

351 KREDAGEYQCEISNPVSVRRSNSIKLDIIFDPTQG..GLSDGAIAGIVIG 398
    ||||||:||||||||||.| |:.||||:| |||||  |||:|||||||||
404 KREDAGDYQCEISNPVSFRISHPIKLDVIPDPTQGNSGLSEGAIAGIVIG 453

399 VVAGVALIAGLAYFLYSRKSGGSGS 423
     ||||||||:|||||||||.||:|.
454 SVAGVALIAALAYFLYSRKTGGGSD 478
```

Figure 27. Proposed domain structure for the MHV receptor based on the deduced amino acid sequence. The first loop shown is defined by a salt bridge formed by an arginine and aspartic acid residue. The three cysteine loops are shown as well as the number of amino acids within and between each loop. The putative transmembrane domain is designated by a filled bar.

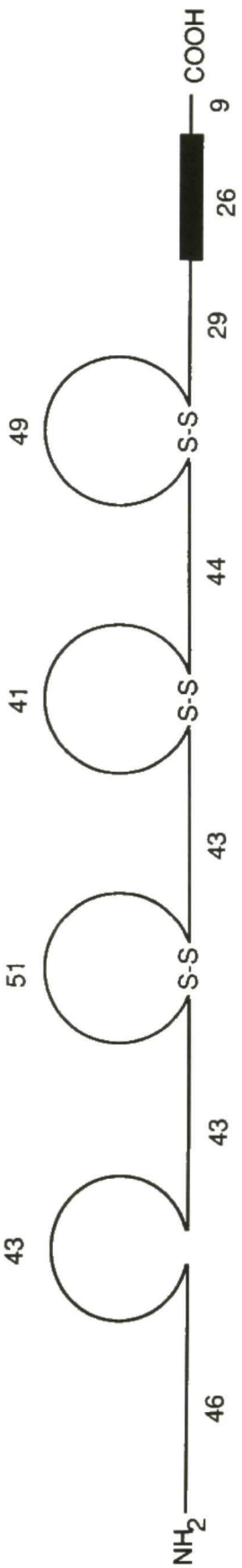
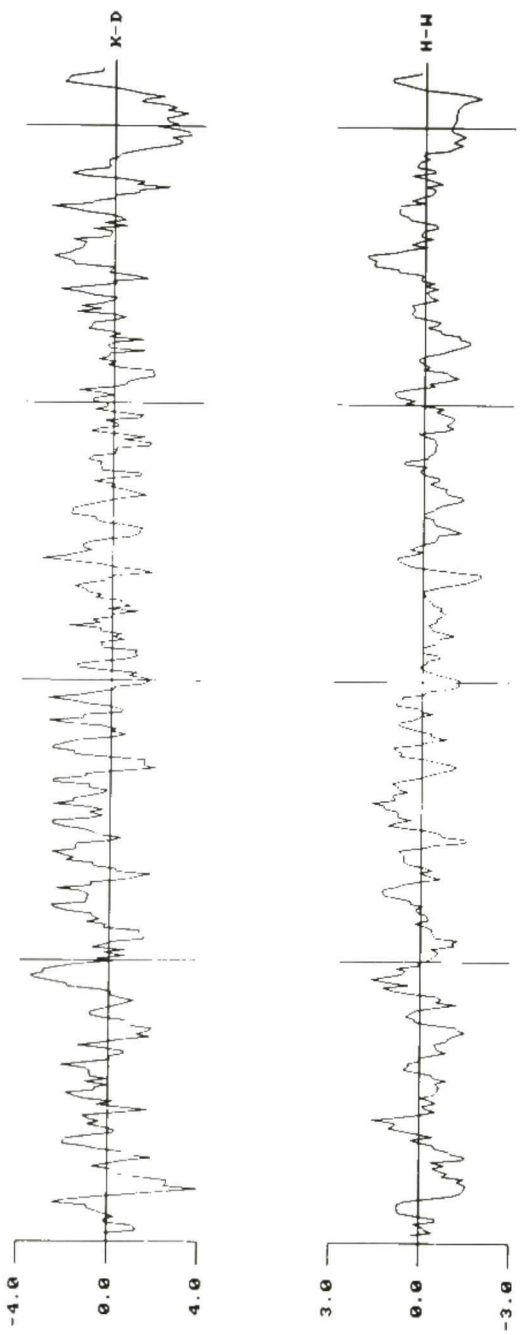

COOH
9
26
29
49
S-S
44
41
S-S
43
51
S-S
43
43
46
NH_2

Figure 28. Predicted structure of MHV receptor polypeptide. The plots show the hydrophobicity analysis according to Kyte-Doolittle (A) and Hopp-Woods (B) of the MHV receptor starting at amino acid 1 of the mature protein.

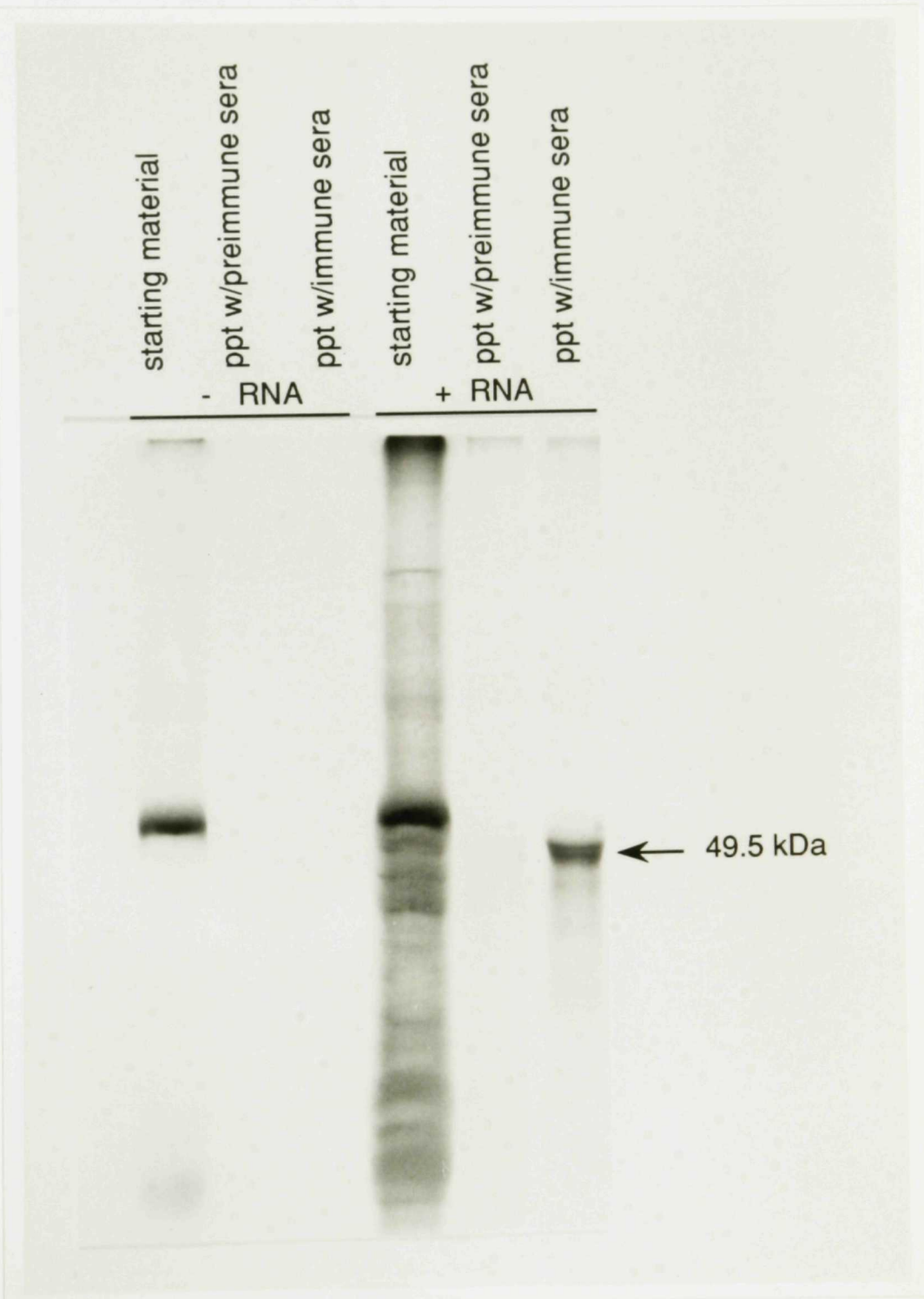

X-D
-4.0
0.0
4.0
H-W
3.0
0.0
-3.0

directly involved in receptor-virus interaction. This conclusion was based on the fact that the MHV- resistant mouse (SJL/J), expresses on its hepatocyte and brush border membranes an homologous protein that is recognized by a polyclonal antibody directed against a synthetic peptide derived from the first 15 amino acids of the affinity purified receptor protein (Williams et al.,1990).

Since the sense primer employed in the RNAPCR reaction is 32-fold degenerate, the base corresponding to the third position of codons 10 to 16 differ between two of the p710 clones sequenced as mentioned above. A single base substitution of an A for a G at amino acid 11 of one of the p710 clones was performed by Dr M. Pensiero by site directed mutagenesis. This changed the original valine for that position to a methionine. The altered clones were screened by hybridization with the mutagenizing oligonucleotide. The selected clone was then sequenced to confirm that the ATG was in frame. The 5' end of this clone containing the new start codon was spliced into pMHVR1 using the Sma I site in the polylinker and the internal Bam HI site at nucleotide 186. This new construct was named pMHVRE and after sequencing the expected nucleotide sequence confirmed. Mutagenesis of that same position in clones pMHVR1 and pMHVR2 was not feasible because of the difference in the nucleotides coding for the valine. While a GTG codon is present in the mutagenized clone p710 a GTT is present in the clones derived from the library. A protein of the correct open reading frame starting from amino acid 11 to the stop codon could be generated after <u>in vitro</u> transcription and translation of this plasmid. An RNA transcript was synthesized from the linearized pMHVRE with

the T7 RNA polymerase by Dr M. Pensiero. The size and integrity of the transcript made was verified by running the material on a formaldehyde gel. In vitro translation of the transcript in a rabbit reticulocyte lysate system in the presence of [^{35}S]- methionine yielded a mixture of labeled proteins. The labeled material was precleared with normal rabbit serum and staphylococcal protein A followed by immunoprecipitation with rabbit polyclonal antibody directed against affinity purified MHV receptor, the monoclonal antibody CC1 or rabbit pre-immune serum. The rabbit polyclonal antibody used in the immunoprecipitation experiments blocks MHV infection of mouse fibroblasts (L_2) at a dilution of 1,200 (Dr G.S. Jiang, personal communication). This antibody is species specific since it fails to recognize brush border membranes of 10 other species (K.V. Holmes, personal communication). As shown in figure 29, a 49,500 Da band was detected in autoradiography of the precipitated material with the polyclonal antibody. This protein was not immunoprecipitated when the RNA transcript was omitted from the in vitro translation reaction or when pre-immune serum or MAb CC1 was employed in the immunoprecipitation. The absence of carbohydrates on the in vitro synthesized molecule and/or the lack of the first 10 amino acids of the mature receptor protein may explain the absence of reactivity with the MAb CC1. The first 10 amino acids might be necessary for the proper folding of the receptor protein. The sugar moieties have been shown to be important for cellular receptor-virus interaction (reported by Dr. Racaniello and Dr. Colonno at the Conference of Microbial Adhesion and Invasion, Panama City, Florida,

Figure 29 . Immunoprecipitation of in vitro translated MHV receptor with rabbit antisera prepared against affinity purified receptor. In vitro transcribed pMHVRE was translated in rabbit reticulocyte lysate as described in the Methods section. Lysates prepared with and without specific mRNA were first pre-cleared with normal rabbit sera, and the incubated with the specific antisera. Following precipitation with Staphylococcal protein A sepharose beads, the bound material was washed extensively and eluted by boiling in sample treatment mix. The eluted material was electrophoresed on a 12 % polyacrylamide gel. The autoradiogram shows the specific precipitation of a 49,500 Da band only with the monospecific antibody directed against the affinity purified MHV receptor glycoprotein.


starting material
ppt w/preimmune sera
ppt w/immune sera
- RNA
starting material
ppt w/preimmune sera
ppt w/immune sera
+ RNA
49.5 kDa

September 1990). Poliovirus does not interact with its cellular receptor unless it is glycosylated, although the carbohydrates are not part of the virus binding domain. The same phenomenon was described for the I-CAM molecule that serves as the cellular receptor for the major rhinovirus group.

DISCUSSION

Recently we confirmed that a murine CEA related glycoprotein serves as the MHV receptor. This result was obtained during the time I was rewriting parts of this dissertation. The full length clone I isolated proved to contain all the information necessary to produce a functional receptor in transient expression assays. Protection studies using polyclonal and monoclonal anti-receptor antibodies had shown that the membrane glycoprotein cloned here is the only functional receptor for the 5 different strains of MHV analyzed. Isolation of the MHV receptor cDNA is the culmination and ultimate validation of the PCR based cloning strategy developed. As with all cloning systems, the ability to isolate the clone is dependent upon a valid probe. For the PCR based cloning systems described here, the N-terminal amino acid sequence (Williams et al., submitted) coupled with the published partial murine CEA cDNA clone (Beauchemin et al.,1989) provided enough information to produce a specific 231 bp and 258 bp products. From the nucleic acid sequence of the p231 clone we were able for the first time to unequivocally state that the MHV receptor is a member of the CEA family of glycoproteins. The next step was to obtain the full length clone coding for the MHV receptor and to identify this clone in the context of the CEA family.

It is interesting to speculate as to why the previous attempts at isolation of the MHV receptor cDNA failed. All screening methods based upon protein expression require that the nucleotide sequence be in the correct orientation

and frame. To date, the four overlapping MHV receptor cDNA clones that have been isolated are out of the proper reading frame with the β-galactosidase fusion system. In addition, the best antisera available at the time of antibody screening was the MAb CC1. So far, MAb CC1 has failed to react with the nonglycosylated receptor protein. This could well be due to a conformational problem or that CC1 binding may be somewhat sugar dependent. The identification of the MAb CC1 binding site as well as its relationship to the virus binding site on the receptor are some of the questions that need to be answered.

The failure of the screening systems based on the degenerate oligonucleotide derived from the N-terminal amino acid sequence is more difficult to explain. The lack of success is most likely due to the combination of two factors. First, the oligonucleotide has a theoretical maximum specific activity that is 6-8 fold lower than a nick translated or random primer labeled probe. Second, there is a low frequency of positive clones containing the full length probe sequence (approximately 0.16×10^{-5}).

Once a cDNA was obtained by screening with the PCR generated p710, its sequence was determined and shown to encode a protein starting at amino acid 10 of the mature receptor protein. This information indicated that the clone (MHVR1.4) was nearly full length. Yet on Northern blot analysis the major RNA species is 3.4 kb, a full 2,000 nucleotides longer. The partial clones obtained are 3' collinear and contain the inefficient poly adenylation signal AGTAAA. New sequence information derived from the full length MHV receptor clone

showed that this 3 kb cDNA contains the same coding region as the previously obtained clones, in addition to 69 bases of 5' untranslated sequence, 102 nucleotides encoding the leader peptide, 27 nucleotides encoding the first 9 amino acids of the mature protein and at least 1.5 kb at its 3'end. This additional 3' sequence is most likely non coding region and includes the consensus poly adenylation signal AATAAA at the 3' end. These facts strongly suggest that the 3.4 kb form is the primary mRNA species and that the 1.7 kb species also detected upon Northern blot analysis arises through the use of the alternate poly adenylation signal detected in the shorter clones we identified. The 3' non coding region of the molecule may play a role in determining the stability of the mRNA. It is interesting to speculate whether this sequence may confer instability on the mRNA in a tissue-specific manner and may be of importance in the normal host cell function of CEA-related proteins. The amount of murine CEA-related glycoprotein present as a consequence of tissue specific expression may be a key determinant in the tissue tropism of MHV.

The MHV receptor full length cDNA clone now available is an important tool to answer several questions related to the first step leading to coronavirus infection. These include the identification of the host cell receptor virus binding domain, the analysis of the structure of the coronavirus-host cell receptor interaction, the correlation between the age of the infected animals and pathogenesis, and the efficacy of antibodies and/or drugs targeted at receptor in controlling MHV-caused disease. A very interesting issue is to define the differences between the receptor glycoprotein of the fully susceptible BALB/c

mice and the homolog expressed in the MHV resistant SJL/J mice strain (Williams et al.,1989). The isolation of the receptor homolog in the resistant strain will aid in defining the virus binding site, the domains of the molecule important in its natural host cell function.

The identification of this CEA-related glycoprotein as the MHV receptor has raised some very interesting questions. First, there is a high degree of homology between related family members of the CEA gene family within a species, the question of whether the other murine CEA related glycoproteins can serve as MHV receptors needs to be addressed. Four murine CEA related glycoproteins isolated from a colon cDNA library have been identified by Beauchemin et al., 1989b. Three of these clones encode the first 25 amino acids identified by microsequencing of the affinity purified MHV receptor. According to their reported sizes and partial sequences, our full length clone is most likely the one defined as mCEA1. However, at this time we can not rule out the possibility that the other two also serve as MHV receptors. Since this gene family in the mouse is not well characterized, the differences in the tissue specific and temporal expression among its members needs to be further explored.

When the secondary structure of human and rat CEA related proteins was analyzed by computer program, it was determined that the CEA family is a member of the immunoglobulin superfamily of glycoproteins (Thompson et al.,1989a). The N-terminal domain of human CEA reveals a close structural similarity to the variable domains of the immunoglobulins, whereas the CEA

"half-repeats" are more similar to the constant domains (Williams, 1987). It is of interest that among the animal virus receptors that have been clearly identified, three have properties of immunoglobulin superfamily cell surface molecules; two are receptors for picornaviruses (poliovirus and the major human rhinovirus) and the third is the receptor for the enveloped retrovirus HIV (Mendelsohn et al., 1989; Greve et al., 1989; Staunton et al., 1989; Tomassini et al., 1989; Klatzmann et al., 1984b; Dalgleish et al., 1984; Maddon et al., 1986).

The identification of the MHV receptor as a member of the family of CEA related glycoproteins has significance in fields besides virology. The function of human (Benchimol, et al.,1989) and mouse (Turbide et al.,1991) CEA has been postulated to be that of an intercellular adhesion molecule in adult and embryonic tissues of the gastrointestinal tract. While CEA may be involved in maintenance of tissue architecture, expression at high levels may also correlate with cell growth. It is important to remember that CEA was first described as a protein overexpressed by adenocarcinomas of the colon and in fetal tissues (Gold and Freedman, 1965), and that characterization of the pattern of expression of the different human CEA family members as well as isolation of all the members of the family is not a closed issue. Human CEA has also been postulated to act as an accesory molecule to the collagen type I receptor in cell-matrix interactions of SW-1222 cells (Pignatelli et al.,1990). The relationship between cell growth, tissue architecture and the neoplastic phenotype is another interesting area of research that will be aided by the cloning of a member of the murine CEA family.

The MHV receptor presents a high degree of homology to the rat CEA related glycoprotein that was reported to have ecto-ATPase activity (Lin and Guidotti, 1989). The function(s) of the rat enzyme ecto-ATPase are not clear at this moment. It has been suggested that the ecto-ATPase may be involved in at least two important cellular functions. One is to hydrolyze extracellular ATP which is a ligand for P_2-purinergic receptors in order to terminate the response (Gordon, 1986), the other possible function is to act as a 5'-nucleotidase for the formation of adenosine and other nucleosides which may either interact with its receptor on the cell surface or may be transported into the cell for the recapture of nucleosides.

The ultimate determination of the cellular function of CEA and its related proteins and their importance in cell biology, development and carcinogenesis are areas of research for many years to come. The characterization of the murine counterparts of CEA strengthens the case for an essential function of this family of glycoproteins and provides a model system for better understanding of the biology of this human tumor marker.

BIBLIOGRAPHY

Acharya, R., E. Fry, D. Stuart, G. Fox, D. Rowlands and F. Brown. (1989). "The three-dimensional structure of foot-and-mouth disease virus at 2.9 A resolution." Nature. **337**: 709-716.

Albritton, L. M., L. Tseng, D. Scadden and J. M. Cunningham. (1989). "A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection." Cell. **57**: 659-666.

Almeida, J. D. and D. A. J. Tyrrell . (1967). "The morphology of three previously uncharacterized respiratory viruses that grow in organ culture." J. Gen. Virol. **1**: 175-178.

Arthos, J., K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A. Truneh, P. J. Maddon and R. W. Sweet. (1989). "Identification of the residues in human CD4 critical for the binding of HIV." Cell. **57**: 469-481.

Atwood, W. J. and L. C. Norkin. (1989). "Class I major histocompatibility proteins as cell surface receptors for simian virus 40." J Virol. **63**: 4474-4477.

Audette, M., F. Buchegger, M. Schreyer and J. P. Mach. (1987). "Monoclonal antibody against carcinoembryonic antigen (CEA) identifies two new forms of crossreacting antigens of molecular weight 90,000 and 160,000 in normal granulocytes." Mol Immunol. **2**: 1177-1186.

Baird, A., Florkiewicz, R. Z., Maher, P. A., Kanner, R. J., and Hajjar, D. P. (1990). "Mediation of virion penetration into vascular cells by association of basic fibroblast growth factor with herpes simplex type I." Nature. **348**: 344-346.

Bang, F. B. and A. Warwick. (1960). "Mouse macrophages as host cells for the mouse hepatitis virus and the genetic basis of their susceptibility." Proc Natl Acad Sci U S A. **46**: 1065-1075.

Barlough, J. E., R. H. Jacobson and F. W. Scott. (1983). "Microtiter assay for coronavirus neutralizing activity in cats using a canine continuous cell line (A-72)." Lab. Anim. Sci. **33**: 567-570.

Barnett, T. R., A. Kretschmer, D. A. Austen, S. J. Goebel., J. H. Hart, J. J. Elting and M. E. Kamarrck (1989). "Carcinoembryonic antigens:alternative splicing accounts for the multiple mRNAs that code for novel members of the carcinoembryonic antigen family." The Journal of Cell Biology. **108**: 267-276.

Barthold, S. W. (1986)."Mouse Hepatitis Virus biology and epizootioloby." pp571-601 Viral and Mycoplasmal infections of laboratory rodents. Effects on Biomedical Research. Orlando, Academic Press,Inc.

Beauchemin, N., C. Turbide, D. Afar, J. Bell, , M. Raymond, C. P. Stanners and A. Fuks. (1989a). "A mouse analogue of the human carcinoembryonic antigen." Cancer Research. **49**: 2017-2021.

Beauchemin, N., C. Turbide, J. Q. Juang, S. Benchimol, S. Jothy, K. Shirota, A. Fuks and C. P. Stanners. (1989 b). Studies on the function of carcinoembryonic antigen pp 49-64. The carcinoembryonic antigen gene family. Amsterdam, Elsevier.

Beaudette, F. R. and C. B. Hudson. (1937). "Cultivation of the virus of infectious bronchitis." J. Am. Vet. Assoc. **90**: 51-60.

Becker, W. B., K. McIntosh, J. H. Dees and R. M. Chanock. (1967). "Morphogenesis of avian infectious bronchitis virus and a related human virus (strain 229E)." J. Virol. **1**: 1019-1027.

Benchimol, S., A. Fuks, S. Jothy, N. Beauchemin, K. Shirota and C. P. Stanners. (1989). "Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule." Cell. **57**: 327-334.

Bennet, J. P., Jr. (1978). Neurotransmitter Receptor Binding. New York, Raven.

Benton, W. D. and R. W. Davis. (1977). "Screening lambda gt recombinant clones by hybridization to single plaques in situ." Science. **196**: 180-182.

Berger, E. A., T. R. Fuerst and B. Moss. (1988). "A soluble recombinant polypeptide comprising the amino-terminal half of the extracellular region of the CD4 molecule contains an active binding site for human immunodeficiency virus." Proc Natl Acad Sci U S A. **85**: 2357-2361.

Binn, L. N., R. H. Marchwicki and E. H. Stephenson. (1980). "Establishment of a canine cell line: derivation, characterization, and viral spectrum." Am J Vet Res. **41**: 855-860.

Bird, A. P. (1986). "CpG-rich islands and the function of DNA methylation." Nature. **321**: 209-213.

Birdwell, C. R. and J. H. Strauss. (1974). "Distribution of the receptor sites for Sindbis virus on the surface of chicken and BHK cells." J Virol. **14**: 672-678.

Bittle, J. L., R. A. Houghten, H. Alexander, T. M. Shinnick, J. G. Sutcliffe, R. A. Lerner, D. J. Rowlands and F. Brown. (1982). "Protection against foot-and-

mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence." Nature. **298**: 30-33.

Blin, N. and D.W. Sttaford. (1976). "Isolation of high-molecular weight DNA." Nucleic Acids Research. **3**: 2303-2308.

Boyle, J. F., D. G. Weismiller and K. V. Holmes. (1987). "Genetic resistance to mouse hepatitis virus correlates with absence of virus-binding activity on target tissues." **61**: 185-189.

Bradburne, A. F. and D. A. J. Tyrrell. (1971). "Coronaviruses of man." Prog. Med. Virol. **13**: 373-403.

Brenner, C. A., A. W. Tam, P. A. Nelson, E. G. Engleman, N. Suzuki, K. E. Fry and J. W. Larrick. (1989). "Message amplification phenotyping (mapping): a technique to simultaneously measure multiple mRNAs from small number of cells." Biotechniques. **7**: 1096-1104.

Brian, D. A., D. E. Dennis and J. S. Guy. (1980). "Genome of porcine transmissible gastroenteritis virus." J Virol. **34**: 410-415.

Brown, M. S. and J. L. Goldstein. (1976). "Analysis of a mutant strain of human fibroblasts with a defect in internalization of receptor-bound low density

lipoprotein." Cell. **9**: 663-674.

Buchegger, F., M. Schreyer, S. Carrel and J. P. Mach. (1984). "Monoclonal antibodies identify a CEA crossreacting antigen of 95 kD (NCA-95) distinct in antigenicity and tissue distribution from the previously described NCA of 55 kD." Int J Cancer. **33**: 643-649.

Burness, A. T. and I. U. Pardoe. (1981). "Effect of enzymes on the attachment of influenza and encephalomyocarditis viruses to erythrocytes." J Gen Virol. **55**: 275-288.

Callebaut, P. and M. Pensaert. (1980). "Characterization and isolation of structural polypeptides in hemagglutinating encephalomyelitis virus." J Gen Virol. **48**: 193-204.

Cardosa, M. J., S. Gordon, S. Hirsch, T. A. Springer and J. S. Porterfield. (1986). "Interaction of West Nile virus with primary murine macrophages: role of cell activation and receptors for antibody and complement." J Virol. **57**: 952-959.

Cavanagh, D. (1984). "Structural characterization of IBV glycoproteins." Adv Exp Med Biol. **173**: 95-108.

Chanh, T. C., G. R. Dreesman and R. C. Kennedy. (1987). "Monoclonal anti-

idiotypic antibody mimics the CD4 receptor and binds human immunodeficiency virus." Proc Natl Acad Sci U S A. **84**: 3891-3895.

Chao, B. H., D. S. Costopoulos, T. Curiel, J. M. Bertonis, P. Chisholm, C. Williams, R. T. Schooley, J. J. Rosa, R. A. Fisher and J. M. Maraganore. (1989). "A 113-amino acid fragment of CD4 produced in Escherichia coli blocks human immunodeficiency virus-induced cell fusion." J Biol Chem. **264**: 5812-5817.

Chatterjee, D. and J. J. Maizel. (1984). "Homology of adenoviral E3 glycoprotein with HLA-DR heavy chain." Proc Natl Acad Sci U S A. **81**: 6039-6043.

Chaudhary, V. K., T. Mizukami, T. R. Fuerst, D. J. FitzGerald, B. Moss, I. Pastan and E. A. Berger. (1988). "Selective killing of HIV-infected cells by recombinant human CD4-Pseudomonas exotoxin hybrid protein." Nature. **335**: 369-372.

Cheever, F. S., J. B. Daniels, A. M. Pappenheimer and O. T. Bailey. (1949). "A murine virus (JHM) causing disseminated encephalomyelitis with extensive destruction of myelin I. Isolation and biological properties of the virus." J Exp Med. **90**: 181-194.

Chomczynski, P. and N. Sacchi. (1987). "Single step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction." Anal. Biochem. **162**: 156-159.

Choppin, P. W. and A. Scheid. (1980). "The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses." Rev Infect Dis. **2**: 40-61.

Clapham, P. R., J. N. Weber, D. Whitby, K. McIntosh, A. G. Dalgleish, P. J. Maddon, K. C. Deen, R. W. Sweet and R. A. Weiss. (1989). "Soluble CD4 blocks the infectivity of diverse strains of HIV and SIV for T cells and monocytes but not for brain and muscle cells." Nature **335**:369-372.

Clarke, M. F., E. P. Gelmann and M. J. Reitz. (1983). "Homology of human T-cell leukaemia virus envelope gene with class I HLA gene." Nature. **305**: 60-62.

Clewell, D. B. and D. R. Helinski. (1972). "Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in Escherichia coli." J Bacteriol. **110**: 1135-1146.

Co, M. S., G. N. Gaulton, A. Tominaga, C. J. Homcy, B. N. Fields and M. I. Greene. (1985). "Structural similarities between the mammalian beta-adrenergic and reovirus type 3 receptors." Proc Natl Acad Sci U S A. **82**: 5315-5318.

Collins, A. R., R. L. Knobler, H. Powell and M. J. Buchmeier. (1982). "Monoclonal antibodies to murine hepatitis virus-4 (strain JHM) define the viral

glycoprotein responsible for attachment and cell-cell fusion." Virology. **119**: 358-371.

Colonno, R. J., P. L. Callahan and W. J. Long. (1986). "Isolation of a monoclonal antibody that blocks attachment of the major group of human rhinoviruses." J Virol. **57**: 7-12.

Compton, S. R. (1988). "Coronavirus Attachment and Replication." The Uniformed Services University of the Health Sciences,Bethesda, MD. Doctoral Dissertation.

Cournoyer, D., N. Beauchemin, D. Boucher, S. Benchimol, A. Fuks and C. P. Stanners. (1988). "Transcription of genes of the carcinoembryonic antigen family in malignant and nonmalignant human tissues." Cancer Res. **48**: 3153-3157.

Craighead, J. E. and M. F. McLane. (1968). "Diabetes Mellitus: induction in mice by Encephalomyocarditis virus". Science. **162**: 913-914

Crowell, R. L. and B. J. Landau. (1983). Receptors in the initiation of picornavirus infections. Comprehensive Virology. New York, Plenum.

Crowell, R. L., B. J. Landau and J.-S. Siak. (1981). Picornavirus receptors in

pathogenesis. Virus Receptors, Part 2 : Animal Virus. London, Chapman and Hall.

Dalgleish, A. G., P. Beverly, P. R. Clapham, D. H. Crawford, M. F. Greaves and R. A. Weiss. (1984). "The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus." Nature. **312**: 763-767.

David-Ferreira, J. F. and R. A. Manaker. (1965). "An electron microscope study of the development of a mouse hepatitis virus in tissue culture cells." J. Cell. Biol. **24**: 57-78.

Deen, K. C., J. S. McDougal, R. Inacker, G. Folena-Wasserman, J. Arthos, J. Rosenberg, P. J. Maddon, R. Axel and R. W. Sweet. (1988). "A soluble form of CD4 (T4) protein inhibits AIDS virus infection." Nature. **331**: 82-84.

DeLarco, J. and G. J. Todaro. (1976). "Membrane receptors for murine leukemia viruses: characterization using the purified viral envelope glycoprotein, gp71." Cell. **8**: 365-371.

Denny, D., Jr., and I. Weissman. (1990). "DNA generated by polymerase chain reaction using Taq DNA polymerase has non-template nucleotide additions: Implications for cloning PCR products". Amplifications. A forum for PCR users. (Perkin Elmer/Cetus). pp. 25-26.

Dieffenbach, C. W., D. N. SenGupta, D. Krause, D. Sawzak and R. H. Silverman. (1989). "Cloning of murine gelsolin and its regulation during differentiation of embryonal carcinoma cells." J Biol Chem. **264**: 13281-13288.

Dindzans, V. J., E. Skamene and G. A. Levy. (1986). "Susceptibility/resistance to mouse hepatitis virus strain 3 and macrophage procoagulant activity are genetically linked and controlled by two non-H2-linked genes." J Immunol. **137**: 2355-2360.

Doyle, L. P. and L. M. Hutchings. (1946). "A transmissble gastroenteritis in pigs." J. Am. Vet. Assoc. **108**: 257-259.

Dubois-Dalcq, M. E., E. W. Doller, M. V. Haspel and K. V. Holmes. (1982). "Cell tropism and expression of mouse hepatitis virus (MHV) in mouse spinal cord cultures." Virology. **119**: 317-331.

Dupuy, J. M., C. Dupuy and D. Decarie. (1984). "Genetically determined resistance to mouse hepatitis virus 3 is expressed in hematopoietic donor cells in radiation chimeras." J Immunol. **133**: 1609-1613.

Dustin, M. L. and T. A. Springer. (1988). "Lymphocyte function associated antigen-1 (LFA-1) interaction with cellular adhesion molecule-1 (ICAM-1) is one

of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells." J.Cell.Biol. **107**: 321-331.

Egan, M. L., D. G. Pritchard, C. W. Todd and V. L. Go. (1977). "Isolation and immunochemical and chemical characterization of carcinoembryonic antigen-like substances in colon lavages of healthy individuals." Cancer Res. **37**: 2638-2643.

Enders, J. F., A. Holloway and E. A. Grogan. (1967). "Replication of poliovirus I in chick embryo and hamster cells exposed to Sendai virus." Proc. Natl. Acad. Sci. USA. **57**: 637-644.

Eppstein, D. A., Y. V. Marsh, A. B. Schreiber, S. R. Newman, G. J. Todaro and J. J. Nestor. (1985). "Epidermal growth factor receptor occupancy inhibits vaccinia virus infection." Nature. **318**: 663-665.

Evermann, J. F., L. Baumgartener, R. L. Ott, E. V. Davis and A. J. McKeirnan. (1981). "Characterization of a feline infectious peritonitis virus isolate." Vet Pathol. **18**: 256-265.

Fingeroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Biro and D. T. Fearon. (1984). "Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2." Proc Natl Acad Sci Usa. **81**: 4510-4514.

Fisher, E. A., J. M. Bertonis, W. Meier, V. A. Johnson, D. S. Costopoulos, T. Liu, R. Tizard, B. D. Walker, M. S. Hirsch, R. T. Schooley and R. A. Flavell. (1988). "HIV infection is blocked in vitro by recombinant soluble CD4." Nature. **331**: 76-78.

Fleming, J. O., S. A. Stohlman, R. C. Harmon, M. Lai, J. A. Frelinger and L. P. Weiner. (1983). "Antigenic relationships of murine coronaviruses: analysis using monoclonal antibodies to JHM (MHV-4) virus." Virology. **131**: 296-307.

Fox, G., N. R. Parry, P. V. Barnett, B. McGinn, D. J. Rowlands and F. Brown. (1989). "The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid)." J Gen Virol. **70**: 625-637.

Freistadt, M. S., G. Kaplan and V. R. Racaniello. (1990). "Heterogeneous expression of poliovirus receptor-related proteins in human cells and tissues." Mol Cell Biol. **10**: 5700-5706.

Fried, H., L. D. Cahan and J. C. Paulson. (1981). "Polyoma virus recognizes specific sialyligosaccharide receptors on host cells." Virology. **109**: 188-192.

Fritsche, R. and J. P. Mach. (1977). "Isolation and characterization of

carcinoembryonic antigen (CEA) extracted from normal human colon mucosa." Immunochemistry. **14**: 119-127.

Frohman, M. A., M. K. Dush and G. R. Martin. (1988). "Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer." Proc Natl Acad Sci U S A. **85**: 8998-9002.

Fukudome, K., O. Yoshie and T. Konno. (1989). "Comparison of human, simian, and bovine rotaviruses for requirement of sialic acid in hemagglutination and cell adsorption." Virology. **172**: 196-205.

Fuller, S. D., C.-H. Bonsdorff and K. Simons. (1985). "Cell surface influenza haemagglutinin can mediate infection by other animal viruses." EMBO J. **4**: 2475-2485.

Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo and M. Popovic. (1986). "The role of mononuclear phagocytes in HTLV-III/LAV infection." Science. **233**: 215-219.

Garwes, D. J., D. H. Pocock and T. M. Wijaskza. (1975). "Identification of heat-dissociable RNA complexes in two porcine coronaviruses." Nature. **257**: 508-510.

Garwes, D. J., D. H. Pocock and B. V. Pike. (1976). "Isolation of subviral components from transmissible gastroenteritis virus." J Gen Virol. **32**: 283-294.

Gilbert, W. (1978). "Why genes in pieces?" Nature. **271**: 501.

Gledhill, A. W. and C. H. Andrews (1951). "A hepatitis virus of mice." Br. J. Exp.Pathol. **32**: 559-568.

Gold, P. and S. O. Freedman. (1965). "Demonstration of tumor-specific antigens in human colonic carcinoma by immunological tolerance and absorption techniques." J Exp Med. **121**: 439-462.

Gordon, J. L. (1986). "Extracellular ATP: effects, sources and fate." Biochem J. **233**: 309-319.

Greve, J. G., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlov, M. E. Kamarck and C. A. Mc. (1989). "The major human rhinovirus receptor is ICAM-1." Cell. **56**: 839-847.

Grundy, J. E., J. A. McKeating, P. J. Ward, A. R. Sanderson and P. D. Griffiths. (1987). "Beta 2 microglobulin enhances the infectivity of cytomegalovirus and when bound to the virus enables class I HLA molecules to be used as a virus receptor." J Gen Virol. **68**: 793-803.

Grunstein, M. and D. S. Hogness. (1975). "Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene." Proc Natl Acad Sci. U S A. **72**: 3961-3965.

Halstead, S. B. (1988). "Pathogenesis of dengue: challenges to molecular biology." Science. **239**: 476-481.

Halstead, S. B. and E. J. O'Rourke. (1977). "Antibody-enhanced dengue virus infection in primate leukocytes." Nature. **265**: 739-741.

Hazelbauer, G. L. (1975). "Role of the receptor for bacteriophage lambda in the functioning of the maltose chemoreceptor of Escherichia coli." J. Bacteriol. **124**: 119-126.

Helenius, A., B. Morein, E. Fries, K. Simons, P. Robinson, V. Schirrmacher, C. Terhorst and J. L. Strominger. (1978). "Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus." Proc Natl Acad Sci U S A. **75**: 3846-3850.

Hinoda, Y., M. Neumaier, A. Hefta, Z. Drzezniek, C. Wagener, L. Shively, L. J. Hefta, J. E. Shively and R. J. Paxton. (1988). "Molecular cloning of a cDNA coding biliary glycoprotein I: Primary structure of a glycoprotein immunologically

crossreactive with carcinoembryonic antigen." Proc Natl Acad Sci Usa. **85**: 6959-6963.

Hirano, N., N. Goto, T. Ogawa, K. Ono, T. Murakami and K. Fujiwara. (1980). "Hydrocephalus in suckling rats infected intracerebrally with mouse hepatitis virus, MHV-A59." Microbiol Immunol. **24**: 825-834.

Hirano, N., S. Takenaka and K. Fujiwara. (1975). "Pathogenicity of mouse hepatitis virus for mice depending upon host age and route of infection." Jpn J Exp Med. **45**: 285-292.

Hogue, B. G. and D. A. Brian. (1986). "Structural proteins of human respiratory coronavirus OC43." Virus Res. **5**: 131-144.

Hogue, B. G., B. King and D. A. Brian. (1984). "Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59." J Virol. **51**: 384-388.

Holland, J. J. (1961). "Receptor affinities as major determinants of enterovirus tissue tropisms in humans." Virology. **15**: 312-326.

Holland, J. J. and L. C. McLaren. (1959). "The mammalian cell: Virus relationship II. Adsorption, reception and eclipse of poliovirus by HeLa cells." J

Exp Med. **109**: 487-504.

Holmes, K. V., J. F. Boyle, D. G. Weismiller, S. R. Compton, R. K. Williams, C. B. Stephensen and M. F. Frana. (1987). "Identification of a receptor for mouse hepatitis virus." Adv Exp Med Biol. **218**: 197-202.

Holmes, K. V., E. W. Doller and J. N. Behnke. (1981a). "Analysis of the functions of coronavirus glycoproteins by differential inhibition of synthesis with tunicamycin." Adv Exp Med Biol. **142**: 133-142.

Holmes, K. V., E. W. Doller and L. S. Sturman. (1981b). "Tunicamycin resistant glycosylation of coronavirus glycoprotein: demonstration of a novel type of viral glycoprotein." Virology. **115**: 334-344.

Holmes, K. V., M. F. Frana, S. G. Robbins and L. S. Sturman. (1984). "Coronavirus maturation." Adv Exp Med Biol. **173**: 37-52.

Holmes, K. V., R. M. Welsh and M. V. Haspel. (1986). "Natural cytotoxicity against mouse hepatitis virus-infected target cells. I. Correlation of cytotoxicity with virus binding to leukocytes." J Immunol. **136**: 1446-1453.

Holmes, K. V. (1989). "Replication of coronaviruses." Virology Vol. 2. pp Fiels B. N. et al., (eds) Raven Press,New York.

Holmes, K. V., R. K. Williams and C. B. Stephensen. "Coronavirus Receptors". Concepts in Viral Pathogenesis III. pp106-113.Edited by A. L. Notkins and M. B. A. Oldstone. Springer-Verlag, New York.

Homsy, J., M. Meyer, M. Tateno, S. Clarkson and J. A. Levy. (1989). "The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells." Science. **244**: 1357-1360.

Hoxie, J. A., B. S. Haggarty, S. E. Bonser, J. L. Rackowski, H. Shan and P. J. Kanki. (1988). "Biological characterization of a simian immunodeficiency virus-like retrovirus (HTLV-IV): evidence for CD4-associated molecules required for infection." J Virol. **62**: 2557-2568.

Hussey, R. E., N. E. Richardson, M. Kowalski, N. R. Brown, H. C. Chang, R. F. Siliciano, T. Dorfman, B. Walker, J. Sodroski and E. L. Reinherz. (1988). "A soluble CD4 protein selectively inhibits HIV replication and syncytium formation." Nature. **331**: 78-81.

Inada, T. and C. A. Mims. (1984). "Mouse Ia antigens are receptors for lactate dehydrogenase virus." Nature. **309**: 59-61.

Ish-Horowicz, D. and J. F. Burke. (1981). "Rapid and efficient cosmid cloning."

Nucleic Acids Res. **9**: 2989-2998.

Itoh, Y., E. Takai, H. Ohnuma, K. Kitajima, F. Tsuda, A. Machida, S. Mishiro, T. Nakamura, Y. Miyakawa and M. Mayumi. (1986). "A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: protective efficacy in chimpanzees." Proc Natl Acad Sci U S A. **83**: 9174-9178.

Jacobsen, H., J. Mestan, S. Mittnacht and C. W. Dieffenbach. (1989). "Beta interferon subtype 1 induction by tumor necrosis factor." Mol Cell Biol. **9**: 3037-3042.

Jameson, B. A., P. E. Rao, L. I. Kong, B. H. Hahn, G. M. Shaw, L. E. Hood and S. B. Kent. (1988). "Location and chemical synthesis of a binding site for HIV- 1 on the CD4 protein." Science. **240**: 1335-1339.

Jeffreys, A. J., V. Wilson, R. Neumann and J. Keyte. (1988). "Amplification of human minisatellites by the polymerase chain reaction: towards DNA fingerprinting of single cells." Nucleic Acids Res. **16**: 10953-10971.

Johnson, R. T. (1965). "Experimental rabies.Studies of cellular vulnerability and pathogenesis using fluorescent antibody staining." Journal of Neuropathology and Experimental Neurology. **24**: 662-674.

Johnson, R. T. (1968). "Mumps virus encephalitis in the hamster.Studies of the inflammatory response and noncytopathic infection of neurons." Journal of Neuropathology and Experimental Neurology. **27**: 80-95.

Jubelt, B. G., O. Gallez-Hawkins, H. Naranyan and R. T. Johnson. (1980). "Pathogenesis of human poliovirus infection in mice.I.Clinical and pathological studies." Journal of Neuropathology and Experimental Neurology. **39**: 138-149.

Kannagi, M., J. M. Yetz and N. L. Letvin. (1985). "In vitro growth characteristics of simian T-lymphotropic virus type III." Proc Natl Acad Sci U S A. **82**: 7053-7057.

Kauffman, R. S., J. H. Noseworthy, J. T. Nepom, R. Finberg, B. N. Fields and M. I. Greene. (1983). "Cell receptors for the mammalian reovirus. II. Monoclonal anti-idiotypic antibody blocks viral binding to cells." J Immunol. **131**: 2539-2541.

Kerem, B.-S., J. M. Rommens, J. A. Buchanan, D. Markiewicz, T. K. Cox, A. Chakravarti, M. Buchwald and L. -C. Tsui. (1990). "Identification of the cystic fibrosis gene: Genetic analysis." Science. **245**: 1073-1080.

Khan, W. N. and S. Hammarstrom. (1989). "Carcinoembryonic antigen gene family: molecular cloning of cDNA for a PS beta G/FL-NCA glycoprotein with a novel domain arrangement." Biochem Biophys Acta. **161**: 525-535.

Khan, W. N., A. Osterman and S. Hammarstrom. (1989). "Molecular cloning and expression of cDNA for a carcinoembryonic antigen-related fetal liver glycoprotein." Proc Natl Acad Sci U S A. **86**: 3332-3336.

King, B. and D. A. Brian. (1982). "Bovine coronavirus structural proteins." J Virol. **42**: 700-707.

King, B., B. J. Potts and D. A. Brian. (1985). "Bovine coronavirus hemagglutinin protein." Virus Res. **2**: 53-59.

Klatzmann, D., F. Barre-Sinoussi, M. T. Nugeyre, C. Danquet, E. Vilmer, C. Griscelli, V. F. Brun, C. Rouzioux, J. C. Gluckman, J. C. Chermann and L. Montagnier. (1984a). "Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes." Science. **225**: 59-63.

Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman and L. Montagnier. (1984b). "T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV." Nature. **312**: 767-768.

Knobler, R. L., L. A. Tunison and M. B. Oldstone. (1984). "Host genetic control of mouse hepatitis virus type 4 (JHM strain) replication. I. Restriction of virus amplification and spread in macrophages from resistant mice." J Gen Virol. **65**:

1543-1548.

Kodelja, V., K. Lucas, S. Barnert, S. Kleist, J. A. Thompson and W. Zimmerman. (1989). "Identification of a carcinoembryonic antigen gene family in the rat. Analysis of the N-terminal domains reveals immunoglobulin-like, hypervariable regions." J Biol Chem. **264**: 6906-6912.

Kohtz, D. S., A. Altman, J. D. Kohtz and S. Puszkin. (1988). "Immunological and structural homology between human T-cell leukemia virus type I envelope glycoprotein and a region of human interleukin-2 implicated in binding the beta receptor." J Virol. **62**: 659-662.

Komai, K., M. Kaplan and M. E. Peeples. (1988). "The Vero cell receptor for the hepatitis B virus small S protein is a sialoglycoprotein." Virology. **163**: 629-634.

Kornfeld, H., N. Riedel, G. A. Viglianti, V. Hirsch and J. I. Mullins. (1987). "Cloning of HTLV-4 and its relation to simian and human immunodeficiency viruses." Nature. **326**: 610-613.

Kozak, M. (1989). "The scanning model for translation: an update." J Cell Biol. **108**: 229-241.

Kuroki, M., Y. Koga and Y. Matsuoka. (1981). "Purification and characterization

of carcinoembryonic antigen-related antigens in normal adult feces." Cancer Res. **41**: 713-720.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature. **227**: 680-685.

Lai, M. and S. A. Stohlman. (1978). "RNA of mouse hepatitis virus." J Virol. **26**: 236-242.

Lai, M. M., C. D. Patton and S. A. Stohlman. (1982). "Further characterization of mRNA's of mouse hepatitis virus: presence of common 5'-end nucleotides." J Virol. **41**: 557-565.

Lai, M. M. C. and S. A. Stohlman. (1981a). "Comparative analysis of RNA genomes of mouse hepatitis viruses." J Virol. **38**: 661-670.

Lai, M. M. C. and S. A. Stohlman. (1981b). "Genome structure of mouse hepatitis virus: comparative analysis by oligonucleotide mapping." Adv Exp Med Biol. **142**: 69-82.

Landau, N. R., M. Warton and D. R. Littman. (1988). "The envelope glycoprotein of the human immunodeficiency virus binds to the immunoglobulin-like domain of CD4." Nature. **334**: 159-162.

Lando, Z., P. Sarin, M. Megson, W. C. Greene, T. A. Waldman, R. C. Gallo and S. Broder. (1983). "Association of human T-cell leukaemia/lymphoma virus with the Tac antigen marker for the human T-cell growth factor receptor." Nature. **305**: 733-736.

Leibowitz, J. L. and S. R. Weiss. (1981). "Murine coronavirus RNA." Adv Exp Med Biol. **142**: 227-243.

Lentz, T. L., T. G. Burrage, A. L. Smith, J. Crick and G. H. Tignor. (1982). "Is the acetylcholine receptor a rabies virus receptor?" Science. **215**: 182-184.

Levy, J. A. (1988). "Mysteries of HIV: challanges for therapy and prevention." Nature. **333**: 519-522.

Lifson, J. D., K. M. Hwang, P. L. Nara, B. Fraser, M. Padgett, N. M. Dunlop and L. E. Eiden. (1988). "Synthetic CD4 peptide derivatives that inhibit HIV infection and cytopathicity." Science. **241**: 712-716.

Lin, S. H. and G. Guidotti. (1989). "Cloning and expression of a cDNA coding for a rat liver plasma membrane ecto-ATPase. The primary structure of the ecto-ATPase is similar to that of the human biliary glycoprotein I." J Biol Chem. **264**: 14408-14414.

Lindsey, J. R. (1986). "Prevalence of viral and mycoplasmal infections in laboratory rodents". pp801-808. Viral and Mycoplasmal infections of Laboratory Rodents. Efects on Biomedical Research. Orlando, Academic Press, Inc.

Loh, E. Y., J. F. Elliott, S. Cwirla, L. L. Lanier and M. M. Davis. (1989). "Polymerase chain reaction with single-sided specificity: analysis of T cell receptor delta chain." Science. **243**: 217-220.

Lomniczi, B. (1977). "Biological properties of avian coronavirus RNA." J Gen Virol. **36**: 531-533.

Lonberg-Holm, K., R. L. Crowell and L. Philipson. (1976). "Unrelated animal viruses share receptors." Nature. **259**: 679-681.

Lonberg-Holm, K. and L. Philipson. (1974). "Early interaction between animal viruses and cells." Monogr. Virol. **9**: 1-149.

Luytjes, W., P. J. Bredenbeek, A. F. Noten, M. C. Horzinek and W. J. Spaan. (1988). "Sequence of mouse hepatitis virus A59 mRNA 2: Indications for RNA recombination between coronaviruses and influenza C virus." Virology. **166**: 415-422.

Mach, J. P. and G. Pusztaszeri. (1972). "Carcinoembryonic antigen (CEA): demonstration of a partial identity between CEA and a normal glycoprotein." Immunochemistry. **9**: 1031-1034.

Machida, A., S. Kishimoto, H. Ohnuma, K. Baba, Y. Ito, H. Miyamoto, G. Funatsu, K. Oda, S. Usuda, S. Togami, T. Nakamura, Y. Miyakawa and M. Mayumi. (1984). "A polypeptide containing 55 amino acid residues coded by the pre-S region of hepatitis B virus deoxyribonucleic acid bears the receptor for polymerized human as well as chimpanzee albumins." Gastroenterology. **86**: 910-918

Macnaughton, M. R., H. A. Davies and M. V. Nermut. (1978). "Ribonucleoprotein-like structures from coronavirus particles." J Gen Virol. **39**: 545-549.

Macnaughton, M. R. and H. M. Madge. (1977). "The characterization of the virion RNA of avian infectious bronquitis virus." FEBS Lett. **77**: 311-313.

Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss and R. Axel. (1986). "The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain." Cell. **47**: 333-348.

Manaker, R. A., Piczak, C.V.m Miller, A.A., and Stanton, M. F. (1961). "A hepatitis complicating studies with mouse leukemia." J.Natl .Cancer Inst. **27**: 29-51.

Mann, D. L., C. E. Read, L. O. Arthur, W. G. Robey, P. Wernet, E. M. Schneider, W. A. Blattner and M. Popovic. (1988). "HLA-DR is involved in the HIV-1 binding site on cells expressing MHC class II antigens ." J Immunol. **141**: 1131-1136.

Margolis, G. and L. Kilham. (1969). "Hydrocephalus in Hamsters, Ferrets, Rats and Mice following inoculation with reovirus type I. II Pathologic studies." Lab. Invest. **21**: 189-198.

Marmur, J. (1961). "A procedure for the isolation of deoxyribonucleic acid from micro-organisms." J. Mol. Biol. **3**: 208-218.

Massalski, A., M. Coulter-Mackie, R. L. Knobler, M. J. Buchmeier and S. Dales. (1982). "In vivo and in vitro models of demyelinating diseases. Comparison of the assembly of mouse hepatitis virus, strain JHM, in two murine cell lines." Intervirology. **18**: 135-146.

Mastromarino, P., C. Conti, P. Goldoni, B. Hauttecoeur and N. Orsi. (1987). "Characterization of membrane components of the erythrocyte involved in vesicular stomatitis virus attachment and fusion at acidic pH." J Gen Virol. **68**: 2359-2369.

Matsuoka, Y., M. Kuroki, Y. Koga, H. Kuriyama, T. Mori and G. Kosaki. (1982).

"Immunochemical differences among carcinoembryonic antigen in tumor tissues and related antigens in meconium and adult feces." Cancer Res. **42**: 2012-2018.

McClintock, P. R., L. C. Billups and A. L. Notkins. (1980). "Receptors for encephalomyocarditis virus on murine and human cells." Virology. **106**: 261-272.

McCray, J. and G. Werner. (1987). "Different rhinovirus serotypes neutralized by antipeptide antibodies." Nature. **329**: 736-738.

McDonnell, M. W., M. N. Simon and F. W. Studier. (1977). "Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels." J. Mol. Biol. **110**: 119-146.

McDougal, J. S., P. J. Maddon, A. G. Dalgleish, P. R. Clapham, D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, R. A. Weiss and R. Axel. (1986). "The T4 glycoprotein is a cell-surface receptor for the AIDS virus." Cold Spring Harb Symp Quant Biol. **2**: 703-711.

McGrath, M. S., G. Tamura and I. L. Weissman. (1987). "Receptor mediated leukemogenesis: murine leukemia virus interacts with BCL1 lymphoma cell surface IgM." J Mol Cell Immunol. **3**: 227-242.

McIntosh, K., W. B. Becker and R. M. Chanock. (1967). "Growth in suckling mouse brain of "IBV-like" viruses from patients with upper respiratory tract disease." Proc. Natl. Acad. Sci. USA. **58**: 2268-2273.

McIntosh, K., A. Z. Kapikian, K. A. Hardison, J. W. Hartley and R. M. Chanock. (1969). "Antigenic relationships among the coronaviruses of man between human and animal coronaviruses." J Immunol. **102**: 1109-1118.

McLaren, L. C., J. J. Holland and J. T. Syverton. (1959). "The mammalian cell : virus relationship; I. Attachment of poliovirus to cultivated cells from primate and non-primate origen." J. Exp. Med. **109**: 475-485.

McLaren, L. C., J. J. Holland and J. T. Syverton. (1960). "The mammalian cell virus relationship. V. Susceptibility and resistance of cells in vitro to infection by coxsackie A9 virus." J. Exp. Med. **112**: 581-594.

Mendelsohn, C. L., E. Wimmer and V. R. Racaniello. (1989). "Cellular receptor for poliovirus: molecular cloning, nucleotide sequence, and expression of a new member of the immunoglobulin superfamily." Cell. **56**: 855-865.

Menezes, J., J. M. Seigneurin, P. Patel, A. Bourkas and G. Lenoir. (1977). "Presence of Epstein-Barr virus receptors, but absence of virus penetration, in

cells of an Epstein-Barr virus genome-negative human lymphoblastoid T line (Molt 4)." J Virol. **22**: 816-821.

Minor, P. D., P. A. Pipkin, D. Hockley, G. C. Schild and J. W. Almond. (1984). "Monoclonal antibodies which block cellular receptors of poliovirus." Virus Res. **1**: 203-212.

Mizukami, T., T. R. Fuerst, E. A. Berger and B. Moss. (1988). "Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of the CD4 molecule defined by site-directed mutagenesis." Proc Natl Acad Sci U S A. **85**: 9273-9277.

Nemerow, G. R., R. A. Houghten, M. D. Moore and N. R. Cooper. (1989). "Identification of an epitope in the major envelope protein of Epstein-Barr virus that mediates viral binding to the B lymphocyte EBV receptor (CR2)." Cell. **56**: 369-377.

Neumaier, M., U. Fenger and C. Wagener. (1985). "Monoclonal antibodies for carcinoembryonic antigen (CEA) as a model system: identification of two novel CEA-related antigens in meconium and colorectal carcinoma tissue by Western blots and differential immunoaffinity chromatography." J Immunol. **135**: 3604-3609.

Neumaier, M., W. Zimmermann, L. Shively, Y. Hinoda, A. D. Riggs and J. E. Shively. (1988). "Characterization of a cDNA clone for the nonspecific cross-reacting antigen (NCA) and a comparison of NCA and carcinoembryonic antigen." J Biol Chem. **263**: 3202-3207.

Neurath, A. R., S. B. H. Kent, N. Strick and K. Parker. (1986). "Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus." Cell. **46**: 429-436.

Niemann, H., and H. D. Klenk. (1981). "Coronavirus glycoprotein E1, a new type of glycoprotein". J. Mol. Biol. **153**:993-1010.

Niemann, H., B. Boschek, D. Evans, M. Rosing, T. Tamura and H. D. Klenk. (1982). "Post-translational glycosylation of coronavirus glycoprotein E1: Inhibition by monensin." EMBO J. **1**:1499-1504.

Ohara, O., R. L. Dorit and W. Gilbert. (1989). "One-sided polymerase chain reaction: the amplification of cDNA." Proc Natl Acad Sci U S A. **86**: 5673-5677.

O'Neill, H. C., M. S. McGrath, J. P. Allison and I. L. Weissman. (1987). "A subset of T cell receptors associated with L3T4 molecules mediates C6VL leukemia cell binding of its cognate retrovirus." Cell. **49**: 143-151.

Parker, J. C. (1980). The possibilities and limitations of virus control in laboratory animals. Animal Quality and Models in Research. New York, Gustav Fischer Verlag.

Paul, R. W. and P. W. Lee. (1987). "Glycophorin is the reovirus receptor on human erythrocytes." Virology. **159**: 94-101.

Paul, R. W., A. H. Choi and P. W. Lee. (1989). "The alpha-anomeric form of sialic acid is the minimal receptor determinant recognized by reovirus." Virology. **172**: 382-385.

Paulson, J. C., J. E. Sadler and R. L. Hill. (1979). "Restoration of specific myxovirus receptors to asialoerythrocytes by incorporation of sialic acid with pure sialyltransferases." J Biol Chem. **254**: 2120-2124.

Paulson, J. C. (1981). Interactions of animal viruses with cell surface receptors. In The Receptors, vol 2, pp. 131-219. Edited by P. M. Conn. Orlando: Academic Press.

Pedersen, N. C., I. Ward and W. L. Mengeling. (1978). "Antigenic relationships of feline infectious peritonitis virus to coronaviruses of other species." Arch Virol. **58**: 45-53.

Peiris, J. S., S. Gordon, J. C. Unkeless and J. S. Porterfield. (1981). "Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages." Nature. **289**: 189-191.

Pert, C. B., J. M. Hill, M. R. Ruff, R. M. Berman, W. G. Robey, L. O. Arthur, F. W. Ruscetti and W. L. Farrar. (1986). "Octapeptides deduced from the neuropeptide receptor-like pattern of antigen T4 in brain potently inhibit human immunodeficiency virus receptor binding and T-cell infectivity." Proc Natl Acad Sci U S A. **83**: 9254-9258.

Pignatelli, M., H. Durbin, and W. F. Bodmer (1990). "Carcinoembryonic antigen functions as an accesory adhesion molecule mediating colon epithelial cell-collagen interactions". Proc Natl Acad Sci U S A. **87**: 1541-1545.

Pritchett, T. J., R. Brossmer, U. Rose and J. C. Paulson. (1987). "Recognition of monovalent sialosides by influenza virus H3 hemagglutinin." Virology. **160**: 502-506.

Rappolee, D. A., D. Mark, M. J. Banda and Z. Werb. (1988). "Wound macrophages express TGF-alpha and other growth factors in vivo: analysis by mRNA phenotyping." Science. **241**: 708-712.

Rappolee, D. A., A. Wang, D. Mark and Z. Werb. (1989). "Novel method for

studying mRNA phenotypes in single or small numbers of cells." J Cell Biochem. **39**: 1-11.

Reiss, J., M. Krawczak, M. Schloesser, M. Wagner and D. N. Cooper. (1990). "The effect of amplification errors on the mismatch analysis of PCR-amplified DNA." Nucleic Acids Research. **19**: 973-978.

Rossmann, M. G., E. Arnold, J. W. Erickson, E. A. Frankenberger, J. P. Griffith, H. J. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry and G. Vriend. (1985). "Structure of a human common cold virus and functional relationship to other picornaviruses." Nature. **317**: 145-153.

Rottier, P., D. Brandenburg, J. Armstrong, B. A. M. van der Zeijst, and G. Warren. (1984). "In vitro assembly of the murine coronavirus membrane protein E1." Adv Exp Med Biol. **173**: 53-64.

Roux, K. H. and P. Dhanarajan. (1990). "A strategy for single site PCR amplification of dsDNA: priming digested cloned or genomic DNA from an anchor-modified restriction site and a short internal sequence [published erratum appears in Biotechniques 1990 Mar;8(3):265]." Biotechniques. **8**: 48-57.

Rudert, F., W. Zimmermann and J. A. Thompson. (1989). "Intra- and interspecies

analyses of the carcinoembryonic antigen (CEA) gene family reveal independent evolution in primates and rodents." J Mol Evol. **29**: 126-134.

Saiki, R. K., T. L. Bugawan, G. T. Horn, K. B. Mullis and H. A. Erlich. (1986). "Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes." Nature. **324**: 163-166.

Sattentau, Q. J. and R. A. Weiss. (1988). "The CD4 antigen: physiological ligand and HIV receptor." Cell. **52**: 631-633.

Schalk, A. F., and M. C. Hawn. (1931). "An apparently new respiratory disease of baby chicks." J. Am. Vet. Med. Assoc. **78**: 413-422.

Schlegel, R., T. S. Tralka, M. C. Willingham and I. Pastan. (1983). "Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site?" Cell. **32**: 639-646.

Schmidt, O. W., M. K. Cooney and G. E. Kenny. (1979). "Plaque assay and improved yield of human coronavirus in human rhabdomyosarcoma cell line." J. Clin. Micro. **9**: 722-728.

Schochetman, G., R. H. Stevens and R. W. Simpson. (1977). "Presence of infectious polyadenylated RNA in the coronavirus avain infectious bronchitis

virus." Virology. **77**: 772-782.

Shapiro, M.B., and P. Senapathy. (1987). RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Research. **15**: 7155-7175.

Shuster, J., D. M. Thomson, A. Fuks and P. Gold. (1980). "Immunologic approaches to diagnosis of malignancy." Prog Exp Tumor Res. **25**: 89-139.

Siddell, S., H. Wege and V. ter Meulen. (1983). "The biology of coronaviruses." J Gen Virol. **64:**761-766.

Smith, D. H., R. A. Byrn, S. A. Marsters, T. Gregory, J. E. Groopman and D. J. Capon. (1987). "Blocking of HIV-1 infectivity by a soluble, secreted form of the CD4 antigen." Science. **238**: 1704-1707.

Smith, M. S., R. E. Click and P. G. Plagemann. (1984). "Control of mouse hepatitis virus replication in macrophages by a recessive gene on chromosome 7." J Immunol. **133**: 428-432.

Sommer, R. and D. Tautz. (1989). "Minimal homology requirements for PCR primers." Nucleic Acids Res. **17**: 6749

Sorensen, O. and S. Dales. (1985). "In vivo and in vitro models of demyelinating disease: JHM virus in the rat central nervous system localized by in situ cDNA hybridization and immunofluorescent microscopy." J Virol. **56**: 434-438.

Sorensen, O., D. Perry and S. Dales. (1980). "In vivo and in vitro models of demyelinating diseases. III. JHM virus infection of rats." Arch Neurol. **37**: 478-484.

Southern, E. (1975). "Detection of specific sequences among DNA fragments separated by gel electrophoresis." J.Mol.Biol. **98**: 503-517.

Spaan, W. J., P. J. Rottier, M. C. Horzinek, B. A. M. van der Zeijst. (1981). "Isolation and identification of virus-specific mRNAs in cells infected with mouse hepatitis virus (MHV-A59)." Virology. **108**: 424-434.

Spriggs, D. R., R. T. Bronson and B. N. Fields. (1983). "Hemagglutinin variants of reovirus type 3 have altered central nervous system tropism." Science. **220**: 505-507.

Springer, G. F., H. G. Schwick and M. A. Fletcher. (1969). "The relationship of the influenza virus inhibitory activity of glycoproteins to their molecular size and sialic acid content." Proc Natl Acad Sci U S A. **64**: 634-641.

Staunton, D. E., V. J. Merluzzi, R. Rothlein, R. Barton, S. D. Marlin and T. A. Springer. (1989). "A cell adhesion molecule, ICAM-1, is the major surface receptor for rhinoviruses." Cell. **56**: 849-853.

Steinman, R. M., I. S. Mellman, W. A. Muller and Z. A. Cohn. (1983). "Endocytosis and the recycling of plasma membrane." J Cell Biol. **96**: 1-27.

Stern, D. F. and S. I. Kennedy. (1980a). "Coronavirus multiplication strategy. I. Identification and characterization of virus specified RNA." J Virol. **34**: 665-674.

Stern, D. F. and S. I. Kennedy. (1980b). "Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome." J Virol. **36**: 440-449.

Stohlman, S. A. and J. A. Frelinger. (1978). "Resistance to fatal central nervous system disease by mouse hepatitis virus strain JHM. I. Genetic analysis." Immunogenetics. **6**: 277-281.

Stohlman, S. A., J. A. Frelinger and L. P. Weiner. (1980). "Resistance to fatal central nervous system disease by mouse hepatitis virus, strain JHM. II. Adherent cell-mediated protection." J Immunol. **124**: 1733-1739.

Stohlman, S. A. and M. M. Lai. (1979). "Phosphoproteins of murine hepatitis

viruses." J Virol. **32**: 672-675.

Strub, K. and P. Walter. (1989). "Isolation of a cDNA clone of the 14-kDa subunit of the signal recognition particle by cross-hybridization of differently primed polymerase chain reactions." Proc Natl Acad Sci U S A. **86**: 9747-9751.

Sturman, L. S. (1981). "The structure and behavior of coronavirus A59 glycoproteins." Adv Exp Med Biol. **142**: 1-17.

Sturman, L. S. and K. V. Holmes. (1977). "Characterization of coronavirus II. Glycoproteins of the viral envelope: tryptic peptide analysis." Virology. **77**: 650-660.

Sturman, L. S. and K. V. Holmes. (1983). "The molecular biology of coronaviruses." Adv Virus Res. **28**: 35-112.

Sturman, L. S. and K. V. Holmes. (1984). "Proteolytic cleavage of peplomeric glycoprotein E2 of MHV yields two 90 K subunits and activates cell fusion." Adv Exp Med Biol. **173**: 25-35.

Sturman, L. S., K. V. Holmes and J. Behnke. (1980). "Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid." J Virol. **33**: 449-462.

Suggs, S. V., R. Bruce Wallace, T. Hiroshe, E. H. Kawashima and K. Itakura. (1981). "Use of synthetic oligonucleotides as hybridization probes: Isolation of cloned cDNA sequences for human beta 2-microglobulin." Proc. Natl. Acad. Sci. USA. **78**: 6613-6617.

Sugiyama, K., R. Ishikawa and N. Fukuhara. (1986). "Structural polypeptides of the murine coronavirus DVIM." Arch Virol. **89**: 245-254.

Superti, F., B. Hauttecoeur, M. J. Morelec, P. Goldoni, B. Bizzini and H. Tsiang. (1986). "Involvement of gangliosides in rabies virus infection." J Gen Virol. **67**: 47-56.

Svenberg, T., S. Hammarstrom and A. Hedin. (1979). "Purification and properties of biliary glycoprotein I (BGP I). Immunochemical relationship to carcinoembryonic antigen." Mol Immunol. **16**: 245-252.

Taguchi, F., S. G. Siddell, H. Wege and V. ter Meulen. (1985). "Characterization of a variant virus selected in rat brains after infection by coronavirus mouse hepatitis virus JHM." J Virol. **54**: 429-435.

Talbot, P. J. and M. J. Buchmeier. (1985). "Antigenic variation among murine coronaviruses: evidence for polymorphism on the peplomer glycoprotein, E2."

Virus Res. **2**: 317-328.

Tardieu, M., R. L. Epstein and H. L. Weiner. (1982). "Interaction of viruses with cell surface receptors." Int Rev Cytol. **80**: 27-61.

Tardieu, M. and H. L. Weiner. (1982). "Viral receptors on isolated murine and human ependymal cells." Science. **215**: 419-421.

Thomas, P. S. (1980). "Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose." Proc.Natl.Acad.Sci. USA. **77**: 5201-5205.

Thompson, J., S. Barnert, B. Berling, S. von Kleist, V. Kodelja, K. Lucas, E. M. Mauch, F. Rudert, H. Schrewe, M. Weiss and W. Zimmermann. (1989a). "Structure, expression and evolution of the human and rat carcinoembryonic antigen (CEA) gene families." Elsevier Science Publishers BV (Biomedical Division). pp. 65-74.

Thompson, J. A., E. M. Mauch, F. S. Chen, Y. Hinoda, H. Schrewe, B. Berling, S. Barnert, S. Kleist, J. E. Shively and W. Zimmerman. (1989b). "Analysis of the size of the carcinoembryonic antigen (CEA) gene family: Isolation and sequencing of N-terminal domain exons." Biochem Biophys Res Comm. **158**: 996-1004.

Thompson, J. A., H. Pande, R. J. Paxton, L. Shively, A. Padma, R. L. Simmer, C. W. Todd, A. D. Riggs and J. E. Shively. (1987). "Molecular cloning of a gene belonging to the carcinoembryonic antigen gene family and discussion of a domain model." Proc Natl Acad Sci U S A. **84**: 2965-2969.

Till, M. A., V. Ghetie, T. Gregory, E. J. Patzer, J. P. Porter, J. W. Uhr, D. J. Capon and E. S. Vitetta. (1988). "HIV-infected cells are killed by rCD4-ricin A chain." Science. **242**: 1166-1168.

Tindall, K. R., and T. A. Kunkel. (1988). " Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase." Biochem. **27**: 6008-6013.

Tomassini, J. E., D. Graham, W. C. De, D. W. Lineberger, J. A. Rodkey and R. J. Colonno. (1989). "cDNA cloning reveals that the major group rhinovirus receptor on HeLa cells is intercellular adhesion molecule 1." Proc Natl Acad Sci Usa. **86**: 4907-4911.

Traunecker, A., W. L:uke and K. Karjalainen. (1988). "Soluble CD4 molecules neutralize human immunodeficiency virus type 1." Nature. **331**: 84-86.

Tschachler, E., V. Groh, M. Popovic, D. L. Mann, K. Konrad, B. Safai, L. Eron, V. F. diMarzo, K. Wolff and G. Stingl. (1987). "Epidermal Langerhans cells-a target

for HTLV-III/LAV infection." J Invest Dermatol. **88**: 233-237.

Tsiang, H., S. de la Porte, D. J. Ambroise, M. Derer and J. Koenig. (1986). "Infection of cultured rat myotubes and neurons from the spinal cord by rabies virus." J Neuropathol Exp Neurol. **45**: 28-42.

Turbide, C., M. Rojas, C.P. Stanners and N. Beauchemin (1991). "A mouse carcinoembryonic antigen gene family member is a calcium-dependent cell adhesion molecule." J. Biol. Chem. **266**:309-315.

Tyrrell, D. A. J. and M. L. Byone. (1965). "Cultivation of a novel type of common-cold virus in organ cultures." Br. Med. J. **1**: 1467-1470.

Tyrrell, D. A. J., J. D. Almeida, D. M. Berry, C. H. Cunningham, D. Hamre, M. S. Hofstad, L. Mallucci and K. McIntosh. (1968). "Coronaviruses." Nature. **220**: 650.

Vlasak, R., W. Luytjes, J. Leider, W. Spaan and P. Palese. (1988). "The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylesterase activity." J Virol. **62**: 4686-4690.

von Kleist, S., G. Chavanel and P. Burtin. (1972). "Identification of an antigen from normal human tissue that cross reacts with the carcinoembryonic antigen."

Proc. Natl. Acad. Sci. USA. **69**: 2492-2496.

Watanabe, R., H. Wege and V. ter Meulen. (1987). "Comparative analysis of coronavirus JHM-induced demyelinating encephalomyelitis in Lewis and Brown Norway rats." Lab Invest. **57**: 375-384.

Watson, R. (1989). The Formation of Primer Artifacts in Polymerase Chain Reaction. Amplifications. A Forum for PCR Users. (Perkin Elmer/Cetus) pp. 5.

Wege, H., A. Muller and V. ter Meulen. (1978). "Genomic RNA of the murine coronavirus JHM." J Gen Virol. **41**: 217-227.

Wege, H., M. Koga and V. ter Meulen. (1981a). "JHM infections in rats as a model for acute and subacute demyelinating disease." Adv Exp Med Biol. **142**: 327-340.

Wege, H., S. Siddell, M. Sturm and V. ter Meulen. (1981b). "Characterization of viral RNA in cells infected with the murine coronavirus JHM." Adv Exp Med Biol. **142**: 91-101.

Wege, H., S. Siddell, M. Sturm and V. ter Meulen. (1981c). "Coronavirus JHM: characterization of intracellular viral RNA." J Gen Virol. **54**: 213-217.

Wege, H., S. Siddell and V. ter Meulen. (1982). "The biology and pathogenesis of coronaviruses." Curr Top Microbiol Immunol. **99**: 165-200.

Weiner, H. L., K. A. Ault and B. N. Fields. (1980). "Interaction of reovirus with cell surface receptors. I. Murine and human lymphocytes have a receptor for the hemagglutinin of reovirus type 3." J Immunol. **124**: 2143-2148.

Weiner, H. L., D. Drayna, D. J. Averill and B. N. Fields. (1977). "Molecular basis of reovirus virulence: role of the S1 gene." Proc Natl Acad Sci U S A. **74**: 5744-5748.

Weis, W., J. H. Brown, S. Cusack, J. C. Paulson, J. J. Skehel and D. C. Wiley. (1988). "Structure of the influenza virus haemagglutinin complexed with its receptor, sialic acid." Nature. **333**: 426-431.

Weiser, W., I. Vellisto and F. B. Bang. (1976). "Congenic strains of mice susceptible and resistant to mouse hepatitis virus." Proc Soc Exp Biol Med. **152**: 499-502.

Weiss, S. R. and J. L. Leibowitz. (1981). "Comparison of the RNAs of murine and human coronaviruses." Adv Exp Med Biol. **142**: 245-259.

Weiss, S. R. (1983). "Coronaviruses SD and SK share extensive nucleotide

homology with murine coronavirus MHV-A59, more than that shared between human and murine coronaviruses." Virology **126**: 669-677.

Williams, A. (1987). "A year in the life of the immunoglobulin superfamily." Immunol Today. **8**: 298-303.

Williams, R., G. Jiang, S. Snyder, M. Frana and K. V. Holmes. (1990). "Purification of the 110-kilodalton glycoprotein receptor for mouse hepatitis virus (MHV-A59) and identification of a nofunctional homolog in SJL/J mice." J. Virol. **64**: 3817-3823

Wilson, T., D. Papahadjopoulos and R. Taber. (1977). "Biological properties of poliovirus encapsulated in lipid vesicles: antibody resistance and infectivity in virus-resistant cells." Proc Natl Acad Sci U S A. **74**: 3471-3475.

Woyciechowska, J. L., B. D. Trapp, D. H. Patrick, I. C. Shekarchi, P. O. Leinikki, J. L. Sever and K. V. Holmes. (1984). "Acute and subacute demyelination induced by mouse hepatitis virus strain A59 in C3H mice." J Exp Pathol. **1**: 295-306.

Yogo, Y., N. Hirano, S. Hino, H. Shibuta and M. Matumoto. (1977). "Polyadenylate in the virion RNA of mouse hepatitis virus." J Biochem (Tokyo . **82**: 1103-1108.

Yoon, J. W., P. R. McClintock, T. Onodera and A. L. Notkins. (1980). "Virus-induced diabetes mellitus. XVIII. Inhibition by a nondiabetogenic variant of encephalomyocarditis virus." J Exp Med. **152**: 878-892.

Young, R. A. and R. W. Davis. (1983). "Efficient isolation of genes by using antibody probes." Proc. Natl. Acad. Sci. USA. **80**: 1194-1198.

Zimmerman, W., B. Ortlieb, R. Friedrich and S. Kleist. (1987). "Isolation and characterization of cDNA clones encoding the human carcinoembryonic antigen reveal a highly conserved repeating structure." Proc Natl Acad Sci. **84**: 2960-2964.

Zimmermann, W., B. Weber, B. Ortlieb, F. Rudert, W. Schempp, H. H. Fiebig, J. E. Shively, S. von Kleist and J. A. Thompson. (1988). "Chromosomal localization of the carcinoembryonic antigen gene family and differential expression in various tumors." Cancer Res. **48**: 2550-2554.